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ZHILIN LI

# Inflammatory Polarization of Immune Cells in Neurological and Neuropsychiatric Disorders

NEUROSCIENCE CENTER AND
CLINICAL NEUROSCIENCES, NEUROLOGY
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN BIOMEDICINE
UNIVERSITY OF HELSINKI

# Inflammatory Polarization of Immune Cells in Neurological and Neuropsychiatric Disorders

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**ACADEMIC DISSERTATION**

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*To my family*

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# List of original publications

This thesis work is based on the following publications (I-III), which are referred to in the text by their Roman numerals. Original publications are reproduced with the permission of Elsevier.

I. **Li, Z.**, Wei, H., Piirainen, S., Chen, Z., Kalso, E., Pertovaara, A., Tian, L., **2016**. Spinal versus brain microglial and macrophage activation traits determine the differential neuroinflammatory responses and analgesic effect of minocycline in chronic neuropathic pain. **Brain, behavior, and immunity**, 58, 107-117.

II. **Li, Z.**, Ma, L., Kulesskaya, N., Voikar, V., Tian, L., **2014**. Microglia are polarized to M1 type in high-anxiety inbred mice in response to lipopolysaccharide challenge. **Brain, behavior, and immunity** 38, 237-248.

III. **Li, Z.**, Khan., M., Kuja-Panula, J., Guo, D., Chen, Z., Lahesmaa, R., Rauvala, H., Tian, L. AMIGO2 modulates T cell functions and its deficiency in mice ameliorates experimental autoimmune encephalomyelitis. **Submitted.**

The author's contributions to the studies included in this thesis:

I. The author participated in the experimental design, performed the flow cytometry and RT-qPCR experiments, analyzed most of the data, and drafted the manuscript.

II. The author participated in the experimental design, performed the flow cytometry experiment, analyzed most of the data, and helped draft the manuscript.

III. The author participated in the experimental design, performed and analyzed most of the experiments, and drafted the manuscript.

# Abbreviations

| | |
|---|---|
| AMIGO2 | amphoterin-induced gene and open reading frame 2 |
| AMG2KO | Amigo2-knockout |
| Arg1 | arginase 1 |
| ATP | adenosine triphosphate |
| BDNF | brain-derived neurotrophic factor |
| CCL | cysteine-cysteine chemokine ligand |
| CD | cluster of differentiation |
| ConA | concanavalin A |
| CNS | central nervous system |
| DAVID | Database for Annotation, Visualization and Integrated Discovery |
| dpi | days post-immunization |
| DRG | dorsal root ganglia |
| EAE | experimental autoimmune encephalomyelitis |
| EPM | elevated plus maze |
| Fcgr2b | Fcγ receptor 2b |
| GABA | gamma-aminobutyric acid |
| GATA-3 | GATA binding protein 3 |
| GSK-3 | glycogen-synthase kinase 3 |
| IFN | interferon |
| Ig | immunoglobulin |
| IHC | immunohistochemistry |
| IL | interleukin |
| KCC2 | potassium chloride cotransporter |
| KO | knockout |
| LD | light-dark test |
| LPS | lipopolysaccharide |
| LRR | leucine-rich repeat |
| MHCII | major histocompatibility complex class II |
| MOG | myelin oligodendrocyte glycoprotein |
| Mrc1 | mannose receptor, C type 1 |
| mRNA | messenger RNA |
| MS | multiple sclerosis |
| NFAT | nuclear factor of activated T cells |
| NF-kB | nuclear factor kappa B |
| Nos2 | inducible nitric oxide synthase |
| OF | open field test |

| | |
|---|---|
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PFC | prefrontal cortex |
| POD | post-operative day |
| RORγ | RAR-related orphan receptor gamma |
| RRMS | relapse-remitting multiple sclerosis |
| RT-qPCR | quantitative reverse-transcription polymerase chain reaction |
| SC | spinal cord |
| SDH | spinal dorsal horn |
| SNI | spared nerve injury |
| SNRI | serotonin-norepinephrine reuptake inhibitor |
| T-bet | T-box expressed in T cells |
| TCR | T cell receptor |
| Tc | T cytotoxic |
| Th | T helper |
| TNF | tumor necrosis factor |
| Treg | regulatory T cell |
| Trk | tropomyosin receptor kinase |
| WT | wild type |

# Abstract

Neurological disorders and related illnesses are leading causes of disability and suffering, and are major health problems and economic burdens to modern society. There are currently no cures or satisfactory disease-modifying treatments for the majority of neurological diseases and associated comorbid neuropsychiatric symptoms. This is partly due to still limited knowledge of the cellular and molecular mechanisms underlying the etiology and progression of neurological and neuropsychiatric disorders. A substantial body of evidence indicates the involvement of innate or adaptive immune cells and the inflammatory mediators they secrete in various neurological or neuropsychiatric disorders. However, the precise roles that immune cells play and the underlying molecular mechanisms that regulate immune cell functions in these disorders remain largely unknown. The purpose of this thesis work is to explore the role of immune cell inflammatory polarization and their molecular regulatory mechanisms in neurological and comorbid anxiety disorders using several rodent experimental models.

First, we discovered that microglia/macrophages in the brain and spinal cord of naïve rats are different from each other, in terms of their abundancy, inflammatory polarization states, and expression of multiple microglia/macrophage-related immune molecules. Such region-specificity under the steady-state condition may underlie their subsequent opposite inflammatory responses to peripheral nerve injury and the analgesic effect of the microglial/macrophage inhibitor minocycline in chronic neuropathic pain.

Since anxiety disorders are frequently observed in patients with neurological disorders, we further explored the association of brain microglial inflammatory polarization with anxiety traits in mice. We characterized microglia in the brains of four inbred mouse strains (C57BL/6J, FVB/N, DBA/2J, and 129S2/Sv) and discovered a strong positive correlation between brain microglial pro- versus anti-inflammatory (M1/M2) ratio and anxiety-like behaviors in mice. Hence, microglial M1/M2 ratio in the brain may be utilized as an index of anxiety or its regulatory genes as potential drug candidates for treating anxiety disorders.

Finally, we discovered that a cell adhesion molecule AMIGO2 is critically involved in the modulation of T cell and microglial/macrophage functions, particularly T-cell homing and T-helper cell polarization. We also observed that *Amigo2* knockout (AMG2KO) mice exhibited ameliorated EAE. We further demonstrated that *Amigo2*-deficiency in T-helper cells promoted Akt activation and NF-kB and NFAT1 transcriptional activities, thereby leading to elevated T-bet and GATA-3, resulted in increased IFN-γ and IL-10 but decreased IL-17A productions. Therefore, AMIGO2 may be harnessed as a potential diagnostic and therapeutic target for multiple sclerosis.

Taken together, utilizing several naturally existing, surgically, or immunologically induced rodent experimental models for neurological and neuropsychiatric disorders, we discovered that inflammatory polarizations of innate and adaptive immune cells are critically involved in these modeled disorders. Our data suggest that disease-modifying approaches targeting inflammatory polarization of microglia/macrophages or molecules that regulate T-cell polarization (e.g. AMIGO2), may be beneficial for tackling these neurological or neuropsychiatric disorders in the future.

# 1. Introduction

Neurological disorders in the central and/or peripheral nervous systems, e.g. the brain, spinal cord (SC) and peripheral nerves, result in conditions such as neuropathic pain and multiple sclerosis (MS). According to the World Health Organization, hundreds of millions of people worldwide suffer from various neurological disorders and associated comorbidities, such as anxiety, depression, and sleep disturbance (World Health Organization, 2006). These neurological disorders and related illnesses greatly affect both physical and mental health, compromising both working ability and quality of life of patients. Accordingly, neurological disorders are major personal, social, and economic burdens to society.

It is well established that neuronal mechanisms are critically involved in the initiation and progression of neurological or neuropsychiatric disorders, and many pharmacological treatments targeting neuronal mechanisms can, to some extent, alleviate clinical symptoms and improve patient quality of life. However, for most neurological and neuropsychiatric disorders, the current available disease-modifying treatments exhibit low to moderate short-term efficacy, while frequently accompanied with strong side effects and unsatisfactory long-term efficacy. This is largely due to currently limited knowledge of the cellular and molecular mechanisms underlying the development and maintenance of these neurological and neuropsychiatric disorders.

During the past decades, in addition to an improved understanding of neuronal contribution, a growing body of evidence suggests the involvement of innate or adaptive immune cells (e.g. microglia, macrophages and T cells) and the inflammatory mediators that these cells release, in various neurological and neuropsychiatric disorders. However, the precise roles that these immune cells play in neurological and neuropsychiatric disorders remain largely unknown. Furthermore, the molecular mechanisms that modulate the function of these immune cells are even less clear.

In terms of inflammatory properties, microglia/macrophages and T cells are known as the most potent yet versatile cell types in the immune system. In response to subtle changes in their residing microenvironment, these cells can rapidly change their morphological (ramified and amoeboid) or inflammatory (classical pro- and alternative anti-inflammatory polarization) status, or both. In an effort to restore immune homeostasis, these cells may also release various inflammatory mediators and in some circumstances seek help by recruiting other immune cells to combat foreign pathogens or tissue injuries. As the largest immune-privileged organ, the central nervous system (CNS) represents the most challenging environment for maintaining such homeostasis in diseased conditions.

In this thesis work, we used several rodent experimental models to investigate the roles of microglial/macrophage polarization or AMIGO2-mediated T-cell polarization in neurological

and neuropsychiatric disorders including neuropathic pain, MS, and anxiety disorder. We characterized the temporal-spatial roles of microglia/macrophages in the brain and SC and their contributing roles to the analgesic effects of minocycline at different stages following spared nerve injuries (SNI) in rats. Furthermore, we explored the association of microglial inflammatory polarization in the brain with anxiety-like behaviors using four inbred mouse strains with differential anxiety traits. Finally, with an *Amigo2*-knockout (AMG2KO) mouse line, we systemically studied the role of AMIGO2 in modulating T-cell and microglial/macrophage functions, particularly T-cell accumulation and pro- versus anti-inflammatory polarization of T helper (Th) cells, and its involvement in acute experimental autoimmune encephalomyelitis (EAE).

# 2. Review of the literature

## 2.1. Neuropathic pain

### 2.1.1. Overview of neuropathic pain

Acute nociceptive pain is regarded as a highly conserved and adaptive physiological response to external noxious stimuli such that the whole body is alerted and generates appropriate reactions to prevent or limit further damage or injury. However, in certain conditions, such as disease or severe injury, the evoked pain hypersensitivity is unable to return to normal over a protracted time and subsequently progresses into chronic pathological pain.

Among various types of chronic pain, neuropathic pain is the most severe. It is a complex chronic pain state caused by injuries or diseases of the central (brain and SC) or peripheral nervous system. This results in dysfunction of the somatosensory nervous system, ranging from primary nociceptive afferent axons, dorsal root ganglion (DRG), dorsal horn, spinothalamic tract, and up to the supraspinal brain regions (e.g. the brain stem and thalamus) (Benarroch, 2010). In the European population, the prevalence of neuropathic pain was estimated to be 7-8% and 5% might suffer from severe pain symptoms (Bouhassira et al., 2008).

With regard to its etiology, diverse conditions can result in the generation of neuropathic pain. Common causes include cancer, diabetes, herpes zoster and human immunodeficiency virus infection, SC injury, stroke, MS, or as a side effect of cancer chemotherapy or human immunodeficiency virus treatment (Alexander et al., 2014). Clinically, most cases of neuropathic pain are chronic and difficult to treat, and patients frequently develop multiple comorbidities such as anxiety, depression, and sleep disturbance (Scadding and Koltzenburg, 2006), which further contribute to or exacerbate pain sensations (Nicholson and Verma, 2004; Page et al., 2014). Neuropathic pain and associated comorbidities greatly affect working ability and quality of life, and are major health problems in society. Based on the Grading of Recommendations Assessment, Development, and Evaluation, tricyclic antidepressants, anticonvulsants (gabapentin and pregabalin), and serotonin-norepinephrine reuptake inhibitor (SNRI) antidepressants are strongly recommended as the first-line treatments. Lidocaine patches, capsaicin patches and tramadol are second-line therapies, while botulinum toxin A and opioids are third-line therapies for neuropathic pain (Finnerup et al., 2015; Gilron et al., 2015). However, these therapies lack satisfactory long-term efficacies and often have many side effects or safety issues. For instance, tricyclic antidepressants are generally effective in relieving neuropathic pain but may cause blurred vision, constipation, dizziness and stomach upset, and present safety issues for people with heart problems. Compared to tricyclic antidepressants, SNRI antidepressants are much safer and have fewer side effects, but are not as effective for pain relief. Painkillers, particularly opioids, are robust and effective but have strong side effects (e.g. constipation, sedation, and stomach upset). Other topical treatments, such as lidocaine patches, are also

effective, but work the best primarily with localized pain (http://www.webmd.com/pain-management/). Such unsatisfactory drug effects are largely due to the currently limited knowledge on how neuropathic pain is initiated and maintained.

### 2.1.2. Spared nerve injury (SNI) model

To gain insight into the cellular and molecular mechanisms underlying the development and maintenance of neuropathic pain and its comorbidities, a variety of rodent experimental models have been developed. Among these rodent experimental models, the most commonly used are peripheral nerve injury models, including SNI, spinal nerve ligation, chronic nerve constriction injury and spinal nerve lesions adjacent to the DRG (Campbell and Meyer, 2006). Several advantages have facilitated the wide usage of these sciatic nerve injury models in preclinical studies of neuropathic pain. First, the surgical procedures are invasive but easy to execute. Second, the injuries induce reliable, robust, and persistent neuropathic pain-like mechanical hypersensitivity that can be easily measured by the classical von Frey hair test. Finally, only the ipsilateral side of an operated rodent is affected following nerve injuries, and hence the contralateral side may be used as an internal control for experimental purposes (Alexander et al., 2014).

### 2.1.3. Neuropathology in neuropathic pain

With the help of these rodent experimental models, it is now widely appreciated that neuronal mechanisms play a crucial role in central sensitization following peripheral nerve injuries. Current knowledge suggests that after a peripheral nerve injury, a series of changes in nociceptors expressed by DRG neurons take place, such as upregulation of voltage-gated sodium channels and transient receptor potential channels, which result in a reduced threshold of DRG neuronal activation. Repetitive firing of DRG projection neurons triggers the release of multiple signals, including adenosine triphosphate (ATP), brain-derived neurotrophic factor (BDNF), cysteine-cysteine chemokine ligand (CCL)2, L-glutamate and substance P (Benarroch, 2010). These signals then bind to their respective receptors that are expressed on the surface of spinal dorsal horn (SDH) projection neurons (e.g. α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, glutamate, neurokinin-1, and N-methyl-D-aspartate receptors), leading to their activation. Receptor activation could, for instance, reverse the effects of gamma-aminobutyric acid $(GABA)_A$ receptors from inhibitory to excitatory, and thereby triggers SDH neuronal depolarization, calcium influx, and a series of downstream cell signaling events, leading to increased excitability of SDH projection neurons and supraspinal pain sensation (**Fig. 1**) (Benarroch, 2010).

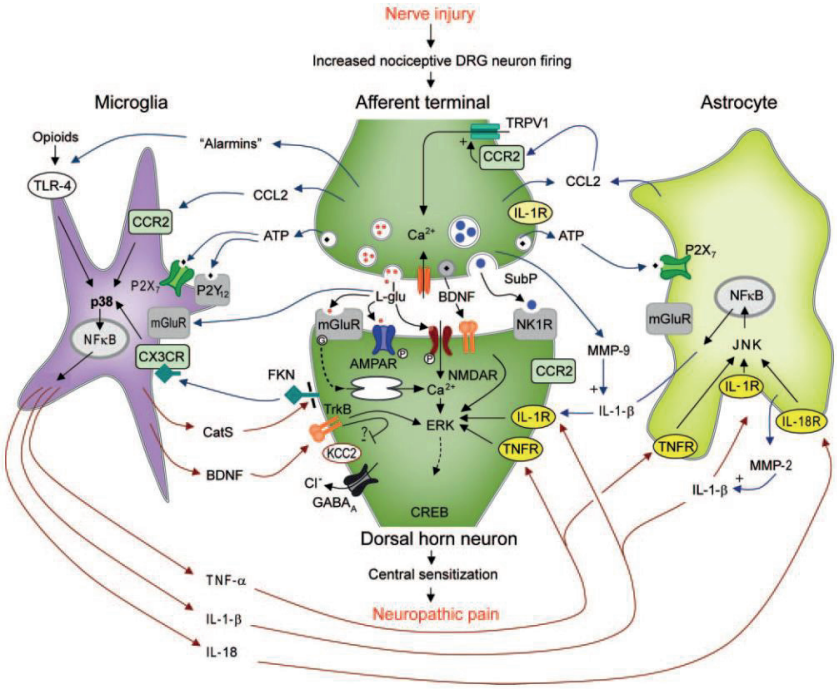


**Figure 1. Neuron-glial interaction in neuropathic pain**. Schematic diagram illustrates the crosstalk between neurons, microglia, and astrocytes following a peripheral nerve injury (**Benarroch, 2010**). Reprinted with permission of Wolters Kluwer Health, Inc via Copyright Clearance Center.

### 2.1.4. Role of infiltrating peripheral immune cells in neuropathic pain

In addition to the well-known neuronal mechanisms, peripheral immune cells that infiltrate in the proximity of the peripheral nerve, or into the DRG or SDH are also implicated in the disease pathogenesis of neuropathic pain. Neutrophils were found in lesioned nerves as early as one hour and peaked within a day, whereas bone-marrow derived macrophages were recruited much later but within 24 hours and peaked in the lesioned nerve at around 1-4 weeks following a peripheral nerve injury (Myers et al., 1996; Perkins and Tracey, 2000). Infiltrating neutrophils and macrophages release highly proalgesic pro-inflammatory substances, including cytokines, reactive oxygen species, and prostaglandins. Pharmacological treatment that suppresses immune responses or depletes circulating neutrophils or macrophages significantly impaired the infiltration of immune cells, reduced axonal degeneration, and attenuated neuropathic pain-like hyperalgesia (Barclay et al., 2007; Bennett, 1999; Clatworthy et al., 1995; Liu et al., 2000; Perkins and Tracey, 2000), demonstrating a critical contribution from peripheral infiltrating innate immune cells.

Besides innate immune cells, infiltrating T cells also contribute to neuropathic pain-like mechanical hypersensitivity in rodents. It was reported that T cells were present in the SDH of rats at one week after SNI and T cell-deficient recombination activating gene 1 (*Rag1*)-null mice developed less severe neuropathic pain-like mechanical allodynia (Costigan et al., 2009). Similarly, another study demonstrated a contributing role of spinal infiltrating $CD4^+$ but not $CD8^+$ T cells in the maintenance of neuropathic pain-like mechanical hypersensitivity (Cao and DeLeo, 2008). $CD4^+$ T cells (also called as Th cells) can be divided into functionally distinct subtypes, such as Th1, Th2 and Th17 (Zhou et al., 2009). Of these subtypes, both Th1 and Th17 cells have demonstrated importance in neuropathic hypersensitivity (Costigan et al., 2009; Moalem et al., 2004). Th1 cells are the main cell source for production of interferon (IFN)-γ (Schoenborn and Wilson, 2007; Schroder et al., 2004), which activates microglia through IFNγR, and inhibition of the IFN-γ signaling pathway attenuated neuropathic mechanical hypersensitivity (Tsuda et al., 2009). Th17 cells predominantly release interleukin (IL)-17A, which is expressed by T cells infiltrating into injured nerve and plays a role in the recruitment, activation, and migration of neutrophils (Chen and O'Shea, 2008; Kleinschnitz et al., 2006; Weaver et al., 2006). More recently, Sorge et al discovered that instead of using a microglia-dependent pathway that is essential for pain perception in male mice, female mice alternatively use infiltrating T cells for generating neuropathic pain-like mechanical hypersensitivity (Sorge et al., 2015), suggesting that different pharmacological intervention strategies targeting neuroimmune mechanisms may be needed for treating neuropathic pain in men versus women.

### 2.1.5. Microglial phenotypical plasticity

#### 2.1.5.1. Discovery and origin of microglia

Microglia are one type of glial cells that are located throughout the CNS. Microglia function as the first-line defense against pathogens or injuries in the CNS and play an important role in restoring homeostasis. Microglia were originally discovered and named by a Spanish scientist Pio Del Rio-Hortega, one student of Santiago Ramón y Cajal, in the 1920s. After a long period of silence, in 1988 Hickey and Kimura demonstrated that perivascular microglia in the CNS were derived from the bone marrow and were fully competent antigen-presenting cells (Hickey and Kimura, 1988), which further supported Pio Del Rio-Hortega's speculation that microglia and peripheral macrophages share similar functionalities in terms of antigen presentation and phagocytosis. Since then, microglial involvement in CNS development, aging, neurological disorders, and brain inflammatory diseases have been extensively studied.

Originally thought to be derived from hematopoietic stem cells in the bone marrow, similar to peripheral macrophages, microglia were later revealed and are now commonly believed to originate from the yolk sac primitive macrophages during embryonic (E)8.5, colonize the

neuroepithelium, expand and occupy the whole CNS under normal conditions (Ginhoux et al., 2010; Ginhoux et al., 2013).

#### 2.1.5.2. Microglial phenotypical plasticity

In the healthy CNS, microglia are a population of highly dynamic cells as opposed to being in a resting state as previously assumed. They regularly scan their residing microenvironment in the CNS with highly motile processes and protrusions to detect subtle changes through a variety of receptors expressed on their cell surface. These receptors include immunoglobulin (Ig) superfamily receptors (e.g. triggering receptors expressed on myeloid cells and Fcγ receptors), chemokine receptors, pattern recognition receptors (e.g. toll-like receptor (TLR)4), purinergic receptors, and phosphatidylserine receptors and gap junction proteins (Hu et al., 2014; Nimmerjahn et al., 2005). Upon detection of danger signals within the residing microenvironment, microglia can make rapid responses by changing their morphology, immunological status (pro- versus anti-inflammation), or both. Such responses depend on the location and function they have to fulfill in combating pathogens or injuries and restoring brain homeostasis.

##### 2.1.5.2.1. Microglial morphological plasticity

Under physiological conditions, microglia exhibit a ramified morphology with extremely motile processes and protrusions (Nimmerjahn et al., 2005). Upon detection of danger signals in the residing microenvironment after internal or external pathological insults or injuries, microglia can rapidly alter their morphology from ramified to amoeboid (or phagocytic) to exert appropriate functions (e.g. phagocytosis of cellular debris). However, recent studies suggest that microglial morphological changes are actually much more complicated than just the bi-modular ramified-amoeboid switches, and more transitional microglial morphological phenotypes exist as proposed in several models (Walker et al., 2014). Notably, Hanisch and Kettenmann proposed that upon activation, microglia can transform into several functionally distinct morphological phenotypes. For example, the reactive phenotypes may transition between different phenotypes depending on the elimination of initial activating signals and feedback signals coming from residing microenvironment within the CNS (Hanisch and Kettenmann, 2007).

##### 2.1.5.2.2. Microglial immunological plasticity

In terms of immunological functions such as antigen presentation and phagocytosis, microglia are considered rather similar to their macrophage counterparts in the peripheral immune system. Microglia and macrophages are regarded as the most potent innate immune cells with diverse immunological phenotypes, with roles in CNS tissue destruction, repair, and regeneration, particularly under pathological conditions (Hu et al., 2015). Similar to the pro-inflammatory M1 versus anti-inflammatory M2 paradigm for peripheral macrophages, microglial M1/M2

polarization has been characterized in a variety of neurological or neuropsychiatric disorders (e.g. neuropathic pain, MS, and anxiety) (Franco and Fernandez-Suarez, 2015; Hu et al., 2015; Li et al., 2014; Miron et al., 2013; Popiolek-Barczyk et al., 2015; Xu et al., 2016). In response to subtle microenvironmental changes, microglia may acquire functionally distinct immune phenotypes to exert diverse effector functions. These immune phenotypes include classically activated M1, alternatively activated M2, and acquired deactivated M2 (**Fig. 2**) (Colton and Wilcock, 2010; Colton, 2009; Frank et al., 2007).

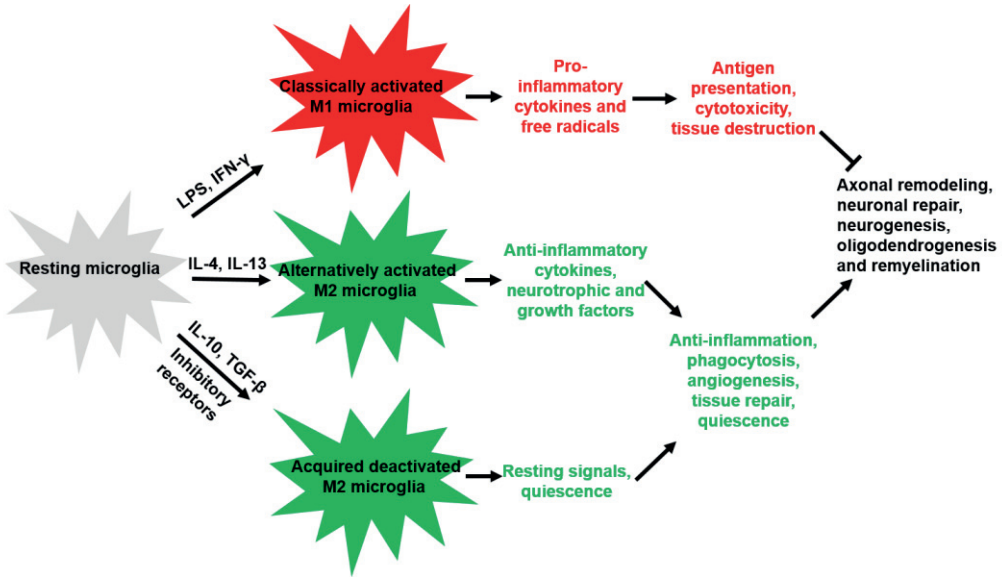


**Figure 2. Microglial M1/M2 paradigm and its role in neurological disorders**. Schematic diagram illustrates different microglial polarization states including classically activated M1, alternatively activated M2, and acquired deactivated M2, and their distinct roles in inflammation and neurovascular network.

Upon stimulation by pro-inflammatory mediators present in the residing microenvironment (e.g. IFN-γ, lipopolysaccharide (LPS), and tumor necrosis factor (TNF)-α), microglia upregulate several co-stimulatory molecules, such as major histocompatibility complex class II (MHCII). Microglia can also secrete pro-inflammatory cytokines and mediators, such as nitric oxide and free radicals, thereby contributing to and exacerbating neuronal tissue damage. In contrast, following stimulation by anti-inflammatory substances (e.g. IL-4, IL-10, and IL-13), microglia can be alternatively activated by upregulating various cell surface receptors for scavenging (e.g. cluster of differentiation (CD)206) and phagocytosis, and secrete anti-inflammatory cytokines (e.g. IL-4, IL-10, and IL-13), growth factors, and neurotrophic factors. These help dampen inflammation, clear cell debris, and promote angiogenesis (Saijo and Glass, 2011). In addition,

microglia may also acquire an M2-like deactivated/quiescent state through constitutively expressing inhibitory cell surface receptors (e.g. CD172a, CX3CR1, and CD200R), which constantly receive contact-dependent signals from their respective neuronal ligands, such as CD47, CX3CL1, and CD200 (Carson et al., 2007; Cherry et al., 2014; Hu et al., 2014; Ransohoff and Cardona, 2010; Saijo and Glass, 2011). Emerging evidence suggests that microglia may upregulate surface expression of such receptors and thus play a neuroprotective role in multiple CNS diseases or injuries (Franco and Fernandez-Suarez, 2015; Hu et al., 2015). For example, CD172a bears an inhibitory immunoreceptor tyrosine-based inhibition motif in its cytoplasmic tail. It has been shown to confer an inhibitory effect on the activation of other inflammatory receptors (Blackbeard et al., 2007; Linnartz and Neumann, 2013).

However, we should bear in mind that pro-inflammatory M1 and anti-inflammatory/quiescent M2 microglia are just two facets of a complicated activation profile, which represent a simplified model in understanding the immune functions of microglia. More polarization states of microglia exist, particularly *in vivo* as recently proposed (Heppner et al., 2015). As such, nowadays some researchers do not favor the most commonly used M1/M2 paradigm for microglia (Ransohoff, 2016). Nonetheless, the concept of the simplified microglial M1/M2 paradigm remains as a useful starting point to define the detrimental or beneficial role of microglial activation and to screen novel pharmacological interventions that target microglia in various neurological and neuropsychiatric disorders.

#### 2.1.5.3. Microglial regional specificity

For a rather long time, researchers have considered microglia as a homogeneous glial cell population throughout the CNS and therefore have mistakenly assumed that they play exactly the same roles regardless of their location within the CNS. Based on this misleading assumption and limited availability of spinal microglia, most researchers, especially those investigating spinal microglia (e.g. SC injury and neuropathic pain), have used brain-derived microglia as their study materials to understand possible involvement of spinal microglia in these disorders or injuries (Bronstein et al., 2013).

Although microglia were initially shown to be differentially distributed within various brain regions of adult rodents (Lawson et al., 1990; Savchenko et al., 1997), it was not until recently that the CNS region-specific feature of microglia began to be appreciated. Several studies, including our own, have shown that microglia are actually very different within different CNS regions, in terms of their abundancy, morphology, and molecular features (Doorn et al., 2015; Grabert et al., 2016; Lawson et al., 1990; Li et al., 2016; Olson, 2010; Savchenko et al., 1997). Nevertheless, it is still unclear what biological effects such microglial regional differences may render on neurons and brain functions in healthy or diseased conditions. An exemplar

interpretation of this feature was recently suggested for aging-related brain function (Grabert et al., 2016).

### 2.1.6. Role of microglia in neuropathic pain

Other than infiltrating immune cells, accumulating evidence suggests that local glial cells substantially modulate neighboring neuronal activity, thereby contributing to neuropathic pain. Indeed, glial cells, astrocytes and microglia included, and neuron-glia interactions are critically involved in the development and maintenance of neuropathic pain (Benarroch, 2010; Grace et al., 2014; Graeber and Christie, 2012).

The possible involvement of microglia in neuropathic pain was demonstrated by animal studies showing efficacy of pharmacological interventions (e.g. minocycline and fluorocitrate) that target microglia on attenuating neuropathic pain-like mechanical allodynia or hyperalgesia (Milligan and Watkins, 2009). Although the compounds were subsequently revealed to target other cell types as well (for example, in addition to microglia, minocycline inhibits macrophages and fluorocitrate targets astrocytes), these studies nevertheless indicate a potential involvement of microglia in the disease pathogenesis of neuropathic pain.

Microglia are central sensors within the CNS and may exhibit a range of reactions following peripheral nerve injury in rodents. These reactions include migration, proliferation, cell body hypertrophy, gene expression, and changes in expression and secretion of proteins. Morphological changes of microglia, to some extent, are considered informative. However, the lack of association between microglial morphological changes and pain hypersensitivity restricts its utility as a useful means to evaluate the contribution of microglia to neuropathic pain (Alexander et al., 2014).

Immunologically, microgliosis and microglial activation represented by upregulation of CD11b/c (integrin alpha M and alpha X chains) take place in early stages following peripheral nerve injury in rodents, accompanied by development of mechanical hypersensitivity (Echeverry et al., 2008). Moreover, pharmacological interventions that inhibit microglial activation are capable of attenuating neuropathic pain-like mechanical hypersensitivity (Ledeboer et al., 2005; Raghavendra et al., 2003). Based on these observations, microglial activation is mainly regarded as a detrimental factor in neuropathic pain. However, there is no direct experimental evidence supporting such a pro-inflammatory role of spinal microglia, since peripheral nerve injury-activated spinal microglia neither express increased levels of MHCII, nor produce large amounts of pro-inflammatory mediators, such as cytokines and reactive oxygen species (Alexander et al., 2014). Therefore, it is possible that instead of playing a pro-inflammatory role, spinal microglia alternatively exhibit neuroprotective phenotypes in neuropathic pain.

Mechanistically, following peripheral nerve injury, microglia can detect multiple signals released by pre-synaptic SDH neurons through a series of receptors expressed on the cell surface. These receptors include pattern recognition receptors (e.g. TLR4), chemokine receptors (e.g. C-C motif chemokine receptor (CCR)20, CX3C chemokine receptor 1 (CX3CR1)), and purinergic receptors (e.g. P2X purinoceptor 4 (P2X4), P2X7, and P2Y12) (**Fig. 1**). Activation of these receptors initiate a cascade of cell signaling events, leading to activation of nuclear factor kappa B (NF-kB), thereby promoting transcription and synthesis of pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. These pro-inflammatory cytokines may activate cyclic adenosine monophosphate (cAMP) response element-binding protein via extracellular signal-regulated kinase signaling pathway by binding to their receptors (e.g. IL-1R, TNFR) expressed on post-synaptic neurons, resulting in potentiation of glutamate-mediated excitation of these neurons. Meanwhile, several other mediators released by microglia are also critical for neuropathic pain hypersensitivity. For example, Cathepsin S can cleave fractalkine and promote its release, which in turn further activates microglia through CX3CR1 receptors. BDNF, another mediator released due to microglial P2X4R activation, may act through tropomyosin receptor kinase B (TrkB) receptor to downregulate the expression of potassium chloride cotransporter (KCC)2 on dorsal horn neurons. The downregulation of KCC2 flips $GABA_A$ receptor from inhibitory to excitatory. Together with neuronal-derived calcium influx and the other above-mentioned signals, these cell-signaling events result in increased excitability of SDH projection neurons and supraspinal pain sensation (Alexander et al., 2014; Benarroch, 2010).

## 2.2. Multiple sclerosis (MS)

### 2.2.1. Overview of MS

MS is the most common chronic inflammatory demyelinating disease in humans. It affects around 2.5 million people worldwide and leads to neurological and physical disability in young adults between the ages of 20-40, with a prevalence rate two-fold higher among women than men (Dendrou et al., 2015). According to the pattern of clinic progression of the disease, MS is subdivided into four major forms, including relapse-remitting (RRMS), progressive relapsing, primary progressive, and secondary progressive types. About 85% of MS patients repeatedly undergo relapses with partial or complete recoveries (or remissions), and hence belong to the RRMS type. More than half of RRMS patients may develop further into secondary progressive MS (Constantinescu et al., 2011). Currently, there is no cure available for MS. However, several disease-modifying treatments are available to lower the relapse rate and slow down the formation of new lesions in RRMS patients. The most commonly prescribed first-line drugs for RRMS patients include type I IFN-β and glatiramer acetate, both of which confer versatile anti-inflammatory, neuroprotective, and regenerative effects (Constantinescu et al., 2011).

Natalizumab (a monoclonal antibody directed against very late antigen-4, which is an integrin essential in the processes of adhesion to endothelium and extravasation by which immune cells, particularly T cells, penetrate into the blood-brain-barrier) and general immunosuppressants (e.g. mitoxantrone and azathioprine) are prescribed as second-line therapies (Constantinescu et al., 2011). However, almost all of these medications are modestly effective accompanied with strong side effects, poor tolerance, or significant life-threatening risks. For instance, IFN-β and glatiramer acetate are injected under the skin or intramuscularly, and may cause side effects such as injection-site reactions, flu-like symptoms, and liver damage (http://www.mayoclinic.org/). Although natalizumab is the most potent disease-modifying treatment, it also increases the risk of an opportunistic infection with latent John Cunningham virus in the brain, leading to progressive multifocal leukoencephalopathy (Constantinescu et al., 2011). General immunosupressants, such as mitoxantrone and azathioprine, generate satisfactory treatment efficacy for certain groups of patients with MS, but may be harmful to the heart and cause blood cancers (http://www.mayoclinic.org/). Therefore, a better understanding of the cellular and molecular mechanisms that critically contribute to the initiation and progression of MS is called for.

### 2.2.2. Experimental autoimmune encephalomyelitis (EAE) disease model

To understand the cellular and molecular mechanisms of MS, a variety of demyelination- and inflammation-induced rodent experimental models have been developed. Among them, the most widely used one is EAE. As a prototype for T-cell-mediated autoimmune disease model, EAE is a complex condition in which various immuno- and neuropathological mechanisms interact with each other and consequently lead to clinical manifestations of key pathological features of MS, namely inflammation, demyelination, gliosis, and axonal damage or loss. In addition, resolution of inflammation and a certain degree of spontaneous remyelination occur in both MS and EAE diseases. Therefore, EAE serves as an excellent rodent experimental model to recapitulate these pathological processes of MS. There are several different ways to induce EAE in rodents. For example, EAE can be induced actively by immunizing animals with myelin-specific antigens, such as myelin oligodendrocyte glycoprotein (MOG), myelin proteolipid protein, and myelin basic protein or their respective peptides, emulsified in complete Freund's adjuvant. Alternatively, EAE can be passively induced by adoptive transfer of myelin-reactive T cells generated from donor rodents following active EAE induction (McCarthy et al., 2012).

### 2.2.3. Inflammation in EAE/MS

The fact that EAE can be induced with myelin-specific proteins or their peptides and passively induced by adoptive transfer of myelin-specific T cells clearly suggest that EAE is an immune-initiated disease. In the disease pathogenesis of EAE and MS, almost all adaptive and innate immune cells have been found in demyelinating lesions and implicated to play a role (**Fig. 3**)

(Constantinescu et al., 2011; Lassmann et al., 2012). Following immunization with myelin-specific antigens, dendritic cells are activated in the draining lymph nodes and present myelin antigens to naïve T cells. The primed T cells express and secrete multiple inflammatory factors, such as pro-inflammatory cytokines, which then upregulate the expression of chemokines and integrins, compromising the permeability of the blood-brain barrier and promoting infiltration of various types of immune cells. Upon encountering cognate myelin antigens in the CNS, infiltrating T cells are reactivated by local antigen-presenting cells (e.g. microglia), thereby secreting inflammatory mediators, such as CCL2, IFN-γ, and macrophage inflammatory protein-1, which further promote the recruitment of other peripheral immune cells to the site of inflammation. Meanwhile, microglial activation triggers astrocyte activation. Within the CNS, glial cells and infiltrating immune cells can produce and release various types of neurotoxic agents, including osteopontin, glutamate, nitric oxygen, proteases, and antibodies, which jointly result in axonal loss and myelin damage, followed by neurological impairment and clinical paralysis of experimental animals (Constantinescu et al., 2011; Fletcher et al., 2010).

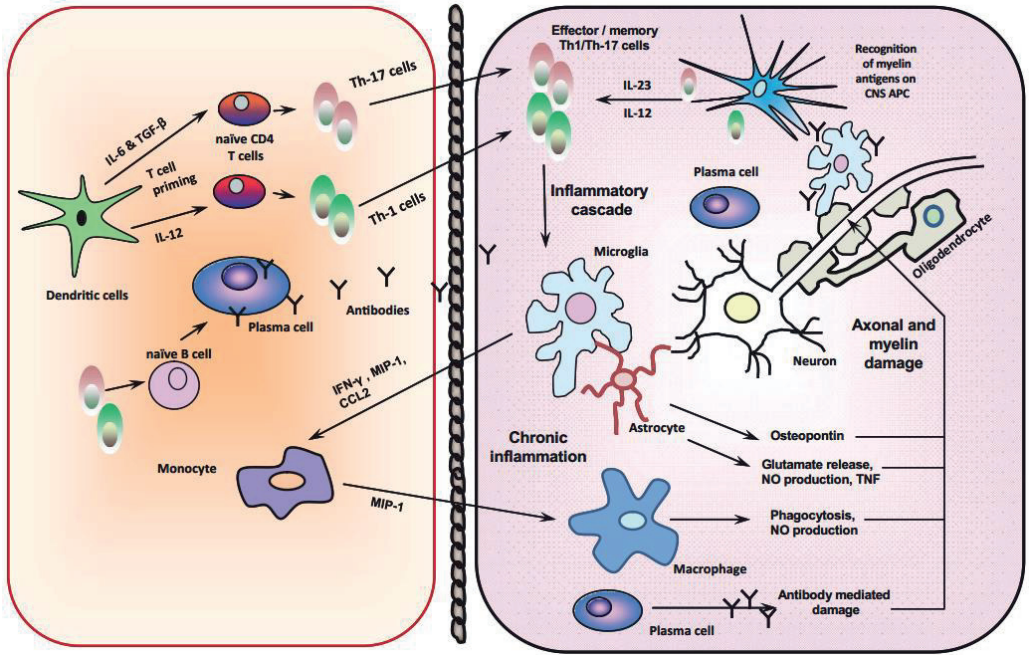


**Figure 3. Roles of immune and glial cells in EAE pathogenesis.** Schematic diagram illustrates the involvement of different immune and glial cells, as well as their interactions in the disease pathogenesis of EAE (**Constantinescu et al., 2011**). Red rectangle: outside of CNS; black rectangle: CNS; the middle line between the rectangles represents the blood-brain-barrier. Reprinted with permission of John Wiley and Sons via Copyright Clearance Center.

### 2.2.4. Role of T cells in EAE/MS

Although the mechanisms that initiate MS remain largely unclear, EAE studies demonstrate a critical involvement of peripheral infiltrating T cells in mediating the pathology (Fletcher et al., 2010). The key role of T cells in MS/EAE was further emphasized by studies showing the success of a pharmacological intervention (Natalizumab) that inhibits T-cell infiltration into the CNS in both preclinical EAE studies and treating RRMS patients (Kappos et al., 2007; Yednock et al., 1992).

In EAE, most of the infiltrating T cells are Th cells, which are known to exhibit versatile immune phenotypes. In response to subtle microenvironmental changes, naïve Th cells are activated and differentiate into functionally distinct Th subsets, mainly Th1, Th2, and Th17 cells, which then secrete Th lineage-specific cytokines to exert their respective effector functions (**Fig. 4**). For example, Th1 cells secrete pro-inflammatory cytokines, including IFN-γ, IL-1β, IL-6, and TNF-α, and were originally thought to be the main Th subset that drives autoimmune responses in EAE. However, more recently, IL-17A-producing Th17 cells were demonstrated to play a major pathogenic role in the development and pathogenesis of EAE. Although both Th1 and Th17 cells are pro-inflammatory and can induce EAE alone, it is speculated that they may play temporally distinct functions but in a collaborative manner in disease pathogenesis, based on the observation that Th1 cells infiltrate the CNS at an earlier phase while Th17 cells are recruited later to maintain or further promote disease development (Fletcher et al., 2010). In contrast to the pro-inflammatory and autoimmunity-promoting roles played by Th1 and Th17 cells, Th2 cells alternatively secrete anti-inflammatory cytokines and mediators, such as IL-4, IL-5, IL-10, and IL-13, which contribute to resolution of inflammation, phagocytosis, angiogenesis, and tissue repair. Therefore, the balance between pro- and anti-inflammatory responses is crucial in determining the initiation and outcome of EAE.

In addition to the contribution from infiltrating Th cells, $CD8^+$ T cytotoxic (Tc) cells are also implicated in the pathogenesis of EAE and MS, but with mixed findings so far. Some studies suggest that Tc cells play a pathogenic role whereas others show that Tc cells are neuroprotective in EAE (Weiss et al., 2007). Other than Th and Tc cells, regulatory T (Treg) cells play a regulatory role in maintaining immune homeostasis during EAE. The importance of Treg cells is further demonstrated by the efficacy of Treg- and IL-10-promoting medications (e.g. IFN-β and glatiramer acetate) in treating MS patients (Arnon and Aharoni, 2009; Dhib-Jalbut and Marks, 2010).

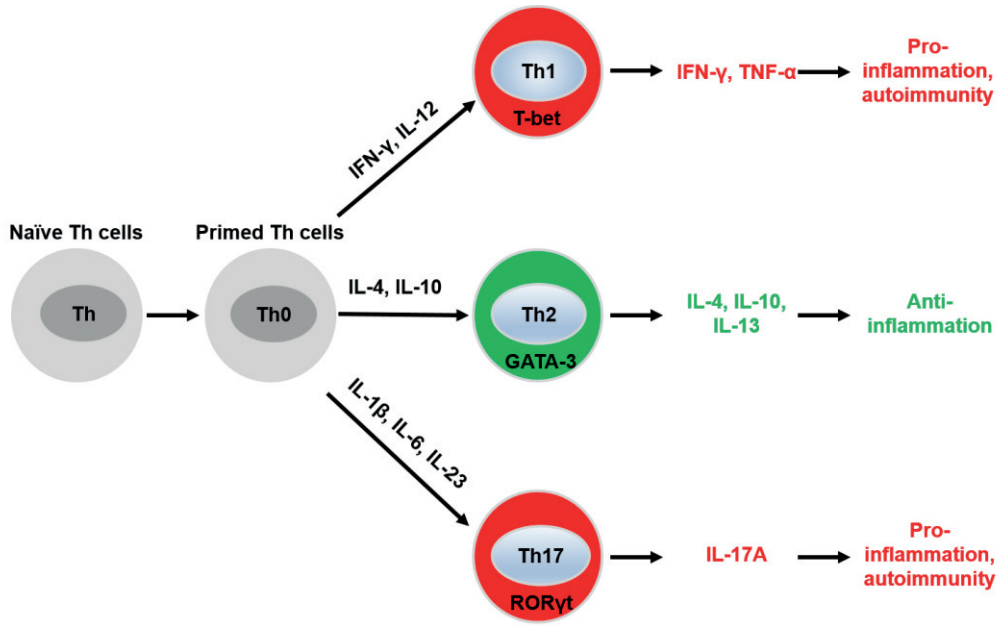


**Figure 4. Th cell differentiation and functionalities.** Schematic diagram illustrates functionally distinct Th cell subtypes, transcription regulators, lineage-specific cytokines, and their effector functions in inflammation and autoimmunity.

In T cell receptor (TCR)-mediated signaling pathways, hallmark lineage-specific transcriptional regulators, such as T-box expressed in T cells (T-bet) and GATA binding protein 3 (GATA-3), are critical in promoting Th-cell differentiation and respective cytokine production. However, detailed molecular mechanisms on how these master transcription factors are regulated in T cells under both physiological and inflammatory conditions are not fully understood (Lazarevic et al., 2013; Tindemans et al., 2014). According to the literature, T cell-specific transcriptional regulators, including nuclear factor of activated T cells (NFAT), NF-kB, activator protein 1 (AP1) and signal transducer and activator of transcription (STAT) proteins, cooperate with each other to drive the transcription and expression of Th lineage-specific transcription factors and cytokine-encoding genes (Hermann-Kleiter and Baier, 2010; Macian, 2005). Among NFAT family members, NFAT1 is the main isoform expressed by T cells (Macian et al., 2002). TCR ligation activates the calcium-calcineurin-NFAT pathway, thereby dephosphorylating NFAT and promoting its nuclear translocation. Once accomplishing its task in the nucleus, dephosphorylated NFAT is re-phosphorylated by maintenance kinases, such as glycogen-synthase kinase 3 (GSK-3) and casein kinase 1, to expose nuclear export signals followed by translocation back to the cytosol (Macian, 2005).

### 2.2.5. Role of microglia/macrophages in EAE/MS

Besides infiltrating T cells, macrophages are another cell type that infiltrates the CNS of EAE animals (Constantinescu et al., 2011). Together with infiltrating macrophages, CNS resident microglia play a critical role in mediating neuroinflammation in EAE and MS. Microglia and macrophages can present myelin antigens to infiltrating T cells, leading to their reactivation. This initiates a cascade of cell signaling events, leading to activation of microglia and macrophages. Similar to peripheral infiltrating Th cells, microglia and macrophages exhibit phenotypic plasticity. On one hand, these cells can secrete various types of pro-inflammatory and neurotoxic agents, such as glutamate, nitric oxygen, osteopontin, proteases, reactive oxygen species and antibodies, which jointly result in the apoptosis, oxidative damage, or both of oligodendrocytes and inflammation-induced neurodegeneration. On the other hand, they may secrete anti-inflammatory, phagocytic, and neurotrophic factors to exert phagocytic and neuroprotective roles (Sonobe and Suzumura, 2014).

In addition to the above-mentioned antigen-presenting, inflammatory, demyelination- and neurodegeneration-promoting mechanisms, activated microglia/macrophages may also be involved in synaptic plasticity by affecting the efficacy of excitatory and inhibitory synapses (Benarroch, 2013). For instance, IL-1β, which is secreted by both activated microglia/macrophages and T cells, promotes glutamatergic and suppresses GABAergic transmission, thereby contributing to impaired cognition and spatial learning deficits (Chiaravalloti and DeLuca, 2008; Dutta et al., 2006; Mandolesi et al., 2010). It is also possible that such a long-term potentiation-like phenomenon during immune attacks in early phases will instead represent a highly adaptive compensational response, which promotes functional recovery to counteract further clinical progression after formation of an MS lesion (Nistico et al., 2014).

### 2.2.6. Neuropathology in MS

Inflammation plays a critical role in the pathogenesis of MS and EAE. The disease-modifying treatments based on the concept of immunopathogenesis have been successfully translated into clinical use for RRMS patients (Kappos et al., 2007). However, the currently available immunosuppressive therapies are largely ineffective in stopping the disease progression of primary and secondary progressive MS patients, even though these treatments confer effects to reduce inflammation and relapses (Constantinescu et al., 2011). These observations suggest that in addition to inflammatory mechanisms, neuropathological mechanisms may also contribute to the disease progression of MS, particularly in progressive MS patients (Lassmann et al., 2012; Stadelmann, 2011). It was shown that in a subset of MS patients, oligodendrocyte apoptosis was the earliest structural change found in all newly forming lesions, with pronounced microglial activation and absence of infiltrating lymphocytes and phagocytes (Matute and Perez-Cerda,

2005). This phenomenon was also found in early RRMS patients in another study (Lucchinetti et al., 2000). Oligodendrocyte apoptosis may be triggered by viral infection (such as human endogenous retrovirus type W (HERV-W)), enhanced extracellular glutamate levels, or other signals produced during hypoxia and oxidative stress (Matute and Perez-Cerda, 2005). Besides oligodendrocyte and myelin damage, emerging evidence suggests that mitochondrial dysfunction is crucial in driving axonal degeneration, thereby contributing to the neurodegenerative processes in MS. Following demyelination, the intra-axonal $Na^+$ concentration is increased due to enrichment of $Na^+$ channels present on axons. Consequently, a greater energy supply and thus more mitochondrial content are required to remove excess intra-axonal $Na^+$ by the $Na^+/K^+$ ATPase. However, due to cortical pathology or inflammatory damage, the $Na^+/K^+$ ATPase and ATP supply are compromised. Increased mitochondrial dysfunction accumulates and this alternatively reverses axonal $Na^+/Ca^{2+}$ pumps, which pumps $Na^+$ out and $Ca^{2+}$ in, leading to $Ca^{2+}$ influx. The rising axonal $Ca^{2+}$ concentration triggers a cascade of cell signaling events, resulting in enhanced production of glutamate and reactive oxygen species, which contribute to axonal degeneration (Witte et al., 2014). Therefore, it is very likely that, at least in some MS patients, a neuropathological mechanism prevails over inflammation and plays a primary role in the disease initiation and progression (Su et al., 2009).

### 2.2.7. Cell adhesion molecule AMIGO2

#### 2.2.7.1. AMIGO protein family

Cell adhesion molecules are transmembrane proteins located on the cell surface and bind to the extracellular matrix or other cells. These proteins are involved in a process called cell adhesion, which is important in many functions, such as maintaining multicellular structure, conferring cell-to-cell signaling and, in case of infections, facilitating pathogenic colonization. There are four classical families of cell adhesion molecules, including integrins, cadherins, selectins, and Ig superfamily.

Amphoterin-induced gene and open reading frame (*Amigo*)*1* was originally found to be induced transcriptionally in primary rat hippocampal neurons upon stimulation by neurite-promoting protein amphoterin (also known as high motility group box 1) (Kuja-Panula et al., 2003). Together with two other gene homologues, *Amigo2* and *Amigo3*, these three AMIGOs constitute a novel family of type I transmembrane proteins, which contain six leucine-rich repeat (LRR)s and one Ig-like domain in their extracellular amino terminus. At the amino acid level, the similarity between AMIGOs is about 50%. In terms of tissue distribution pattern in the adult mouse, *Amigo1* is mainly enriched in the CNS tissues whereas *Amigo2* and *Amigo3* are more widely distributed (Kuja-Panula et al., 2003).

#### 2.2.7.2. Neuronal and immune roles of AMIGO2

AMIGO proteins simultaneously carry LRR and Ig domains in their structures and belong to LRRIG proteins. Through a homologous BLAST search, a total of 36 human LRRIG proteins were identified, including four LINGOs, three NGLs, five SALMs, three NLRRs, three Pals, two ISLRs, three LRIGs, two GPRs, two Adlicans, two Peroxidasin-like proteins, three Trk neurotrophin receptors, an unnamed protein AAI11068, and three AMIGOs (Homma et al., 2009). Some LRRIG molecules are expressed exclusively in the CNS, whereas the majority of proteins are widely distributed (Homma et al., 2009), indicating their broad-spectrum functions in various cell and tissue types. Since both LRR and Ig domains are frequently found in many proteins with diverse functions (Williams and Barclay, 1988), LRRIG proteins carrying both LRR and Ig domains are thus likely involved in a wide spectrum of protein-protein interactions.

Similar to the predominant neuronal involvement of AMIGO1 in dendritic growth and neuronal survival (Chen et al., 2012; Kuja-Panula et al., 2003; Peltola et al., 2011; Peltola et al., 2016; Zhao et al., 2014), most studies concerning AMIGO2 have also focused on its involvement in neuronal functions. For instance, AMIGO2 was shown to inhibit apoptosis and promote survival of cerebellar granule neurons (Ono et al., 2003). Moreover, AMIGO2 expression in the hippocampal CA2 region was demonstrated to be critical in the formation of social memory (Hitti and Siegelbaum, 2014). Haploinsufficiency of *AMIGO2* was suggested to be potentially responsible for abnormal growth and severe mental retardation in humans (Gimelli et al., 2011; Miyake et al., 2004). In addition to its regulatory role in neurons and associated neurological deficits, AMIGO2 modulates functions of endothelial cells and formation of human gastric adenocarcinoma (Park et al., 2015; Rabenau et al., 2004). However, emerging evidence indicates its possible involvement in the immune system. In one study, *Amigo2* was shown to be differentially expressed by $CD4^+CD8^+$ double-positive, $CD4^+$ and $CD8^+$ single-positive thymocytes (Tsukumo et al., 2006). Moreover, human *AMIGO2* messenger RNA (mRNA) was enriched in Th2 cells compared to Th0 and Th1 cells (Lund et al., 2007). These findings nevertheless indicate that AMIGO2 may be involved in T cell function.

## 2.3. Anxiety as a comorbidity in neurological disorders

### 2.3.1. Anxiety disorders as comorbid conditions in neurological patients

Anxiety disorders are characterized by the feeling of stress or fear. Transient feeling of stress or fear is an involuntary daily biological response and is considered beneficial for human beings to take appropriate actions to cope with potential threats or insults, either external or internal. However, unresolved and prolonged feelings of anxiety may result in mental health problems. Anxiety disorders are the most common mental health problems in European countries, affecting approximately 61.5 million people with a 12-month prevalence rate of 14%. Anxiety disorders

and related illnesses cost European countries hundreds of billions of euros annually (Wittchen et al., 2011).

The most frequently observed anxiety disorders in the general population are generalized anxiety disorder, panic disorder, social phobia, and obsessive-compulsive disorder. In the US population, the prevalence rate for generalized anxiety disorder is 3.1%, 2.7% for panic disorder, 8.7% for social phobia and 1.0% for obsessive-compulsive disorder (https://www.adaa.org/). In neurological patients, anxiety can be viewed as a symptom associated with a neurologic disorder, a side effect due to medical treatment, or a comorbid condition. Compared with the general population, the prevalence rates of anxiety disorders in patients with neurological disorders are even higher. For instance, approximately 38% of neuropathic pain patients developed comorbid anxiety in their lifetime, with generalized anxiety disorder (22.5%), panic disorder (7.6%), social phobia (6.1%), and obsessive compulsive disorder (1.8%) (Radat et al., 2013). In a cohort of patients with MS, as many as 35.7% suffered from any kind of anxiety disorder (generalized anxiety disorder: 18.6%; panic disorder: 10%; obsessive disorder: 8.6%) during their lifetime (Korostil and Feinstein, 2007). When treating neurological disorders, much effort has been placed in relieving neurological symptoms, without any recognition or treatment of comorbid conditions, such as anxiety. This is largely based on the assumption that anxiety seen in these neurological patients is merely a normal response to having a neurological disorder. However, if left untreated, comorbid anxiety disorders may significantly contribute to and exacerbate morbidity and mortality in patients with neurological disorders (Davies et al., 2001). Therefore, a better understanding of contributing cellular and molecular mechanisms in comorbid anxiety is warranted in order to improve current treatment strategies for patients with these conditions.

### 2.3.2. Anxiety-like behaviors in mice

To gain insight into human pathological anxiety, a variety of behavioral testing paradigms have been developed for assessing anxiety levels in inbred mouse strains and genetically modified mouse models. The most widely used classic behavioral tests for measuring anxiety-like behaviors in animals include the open field test (OF), elevated plus maze (EPM), and light-dark (LD) tests. Multiple parameters in these tests can be used as indexes of anxiety levels of an animal. For example, the higher percentage of time that an animal spends in the corner of an open field, the more anxious this animal is (Hölter et al., 2011).

### 2.3.3. Critical brain regions associated with anxiety disorders

Combining with various behavioral paradigms, imaging studies have been widely used to understand threat perception, fear acquisition, aversive-affect processing, and the regulation of these processes in both human and animal subjects (Phan, 2015). In preclinical studies using rodent experimental models, several key brain regions closely associated with anxiety have been

revealed. These brain regions include the prefrontal cortex (PFC), amygdala, and hippocampus (Phan, 2015). It has been suggested that these interconnected brain regions form a frontal-limbic circuit involving prefrontal, limbic, and paralimbic areas. However, how these interconnected brain regions communicate with each other, process danger cues, and ultimately produce the feeling of stress or fear is poorly understood. Current knowledge suggests that neurotransmitters, neuropeptides, and neuroendocrine hormones play a role (Phan, 2015).

### 2.3.4. Role of microglia in anxiety disorders

In rodent experimental models, previous studies have shown that both acute and chronic psychological stress may trigger microglia to produce pro-inflammatory cytokines in the brain, such as IL-1β, IL-6, and TNF-α (Blandino et al., 2009; Frank et al., 2007; Nguyen et al., 1998; Tynan et al., 2010; Wohleb et al., 2012). Under physiological conditions, these cytokines may function as mediators that convey information from the immune to the nervous system, thereby exerting adaptive responses to physical or emotional stress by induction of sickness behaviors or disruption of emotional stress responses (Dantzer et al., 2008; Yirmiya and Goshen, 2011). Cytokines injected directly into brain regions may potentiate anxiety-like behaviors in rats (Connor et al., 1998). In fact, cytokines have been implicated in modulation of neuronal activity in brain regions such as the amygdala, hippocampus, hypothalamus, and cerebral cortex (Besedovsky and del Rey, 1996; Elenkov et al., 2000). Moreover, cytokine signaling within the brain has been shown to modulate several critical brain functions. These functions include neurotransmitter metabolism, neuroendocrine signaling, synaptic plasticity, and neural circuitry of mood formation (Salim et al., 2012). However, exacerbated and prolonged immune activation may be detrimental to memory formation, neuronal plasticity, and neurogenesis (Yirmiya and Goshen, 2011). It has been demonstrated that prenatal immune activation may act as an environmental risk factor, and, together with genetic factors, critically contribute to the pathogenesis of neuropsychiatric disorders. Accordingly, pharmacological treatment with the microglial/macrophage inhibitor minocycline can restore working memory and inhibit depression-like behaviors in rodents (Hinwood et al., 2013; Kreisel et al., 2013).

In addition to direct sensation of psychosocial stressors, microglia are also responsive to environmental stimuli, such as peripheral immune activation, either directly or in combination with psychosocial stress. For example, pre-existing stress exposure sensitized LPS-induced cytokine production (Frank et al., 2007; Johnson et al., 2002). Neonatal infection attenuated corticosterone response to an acute stressor (Bilbo et al., 2005; Bilbo and Schwarz, 2012). Maternal immune activation in mice increased the vulnerability of their offspring to deficits in cognition and somatosensory gating in response to foot-shock-induced stress (Giovanoli et al., 2013). Moreover, peripheral innate immune challenge provoked microglial activation and prolonged social withdrawal in socially defeated mice (Wohleb et al., 2012). Therefore,

microglial activation due to psychosocial stressors or environmental stimulation is critical in anxiety-related mental disorders.

Besides the above-mentioned inflammatory functions, emerging evidence suggests that microglia may also play an important role in synaptic pruning. As such, microglial deficit impairs neuroplasticity and neurogenesis, therefore resulting in cognitive deficits and contributing to development of neuropsychiatric disorders, such as anxiety disorders (Aguzzi et al., 2013; Yirmiya and Goshen, 2011). Using mice lacking the chemokine receptor *Cx3cr1*, Zhan and colleagues demonstrated that a primary microglial deficit during the postnatal period resulted in a long-term deficit in synaptic multiplicity in adulthood, which was accompanied by weakened synaptic transmission, reduced functional brain connectivity, impaired social interaction, and increased repetitive behaviors (Zhan et al., 2014). After birth of *Cx3cr1* KO mice, microglia are also crucial in the formation of dendritic spines, as transient loss of microglia led to increased dendritic spines and immature synapses, which was associated with immature brain circuitry (Paolicelli et al., 2011). Moreover, microglia may be directly involved in modulating the strength of glutamatergic synaptic transmission and plasticity in the hippocampal CA1 area under peripheral inflammatory conditions. This may underlie comorbid conditions, such as anxiety disorder and depression, as observed in patients with inflammatory diseases, neurological or neuropsychiatric disorders in which inflammation is involved (Riazi et al., 2015).

However, how microglial activation influences anxiety remains largely unknown. A more thorough understanding of microglial activation and the molecular mechanisms involved in the regulation of microglial activation in these anxiety disorders will help develop better treatment strategies and identify novel pharmacological therapies for patients suffering from anxiety disorders (Bilbo and Schwarz, 2012; Meyer, 2011).

### 2.3.5. Inbred mice as a rodent model for anxiety disorders

It is known that anxiety disorders run in families, suggesting that genetic factors contribute to the risk of developing anxiety disorders. To identify the genes and biological pathways that are involved in anxiety disorders, several experimental approaches on human subjects have been used, including family and twin studies, linkage and association analysis, for detection of copy number variations and rare single nucleotide polymorphisms (Phan, 2015).

Besides genetic contributions, environmental factors are now believed to play a substantial role in the development of anxiety disorders. In recent years, the interaction between genetic variants and environmental exposures has been a major focus in research of the etiology of anxiety disorders. From this point of view, inbred mice serve as an excellent rodent model for such purposes. Firstly, various inbred mouse strains differ significantly from each other in their anxiety-related endophenotypes concerning their locomotor activities, stress responses, learning

skills, and drug responses (Hovatta and Barlow, 2008; Wahlsten et al., 2006). Secondly, inbred mouse strains have distinct but stable genetic backgrounds with remarkably rich publicly available information (Li et al., 2014). Hence, inbred mice provide an excellent experimental tool to study how genetic disposition and peripheral or central immune activation may jointly affect microglial activation and development of anxiety-like behaviors in these animals. In fact, several high-anxiety mouse strains such as DBA/2, 129S1 and BALB/c (in contrast to low-anxiety mouse strains such as C57BL/6J) have been used for genetic, behavioral, and pharmacological research to identify target genes for diagnostic and therapeutic purposes in human anxiety disorders (Hovatta et al., 2005; Sokolowska and Hovatta, 2013).

# 3. Aims of the study

The aim of this thesis work was to investigate the role of pro- versus anti-inflammatory polarizations of microglia/macrophages or AMIGO2-mediated T cells in neurological and neuropsychiatric disorders using several rodent experimental models.

My specific objectives were:

1) To characterize temporal-spatial profiles of microglia/macrophages in the brain and SC during the course of chronic neuropathic pain, and to unravel possible mechanism of efficacy (and inefficacy) of minocycline on attenuating mechanical allodynia following SNI surgery in rats.
2) To explore the association of microglial inflammatory polarization in the brain with anxiety-like behaviors in mice using four inbred mouse strains with different anxiety traits.
3) To study the role of a cell adhesion molecule AMIGO2 in modulating T-cell and microglial/macrophage functions and in acute EAE using an AMG2KO mouse line.

# 4. Materials and methods

The materials and methods used in this thesis are listed below and described in details in the original publications, which are referred in the text by their Roman numerals.

## 4.1. Materials

**Table 1. Mouse and rat strains**

| Mouse/rat strain | Description | Source | Used in |
|---|---|---|---|
| 129S2/Sv mouse | Inbred mouse strain | Envigo | II |
| AMG2KO | *Amigo2*-deficient inbred mouse strain | Laboratory Animal Center at the University of Helsinki | III |
| C57BL/6J mouse | Wild type (WT) inbred mouse strain | Envigo and Laboratory Animal Center at the University of Helsinki | II, III |
| DBA/2J mouse | Inbred mouse strain | Envigo | II |
| FVB/N mouse | Inbred mouse strain | Envigo | II |
| Hannover-Wistar rat | Outbred rat strain | Envigo | I |

**Table 2. Plasmids**

| Plasmids | Description | Source | Used in |
|---|---|---|---|
| pGL3-NF-kB | NF-kB promoter-driven firefly luciferase reporter plasmid | Dr. Guo at Wuhan University | III |
| pRL-TK | Thymidine kinase promoter-driven renilla luciferase reporter plasmid | Promega | III |
| signal-FLAG pIG-Amigo2 | Full length *Amigo2* CDS fused to signal FLAG | Generated by Dr. Kuja-Panula | III |
| signal-FLAG pIG-Amigo2ΔCyto | Cytoplasmid domain-deficient *Amigo2* CDS fused to signal FLAG | Generated by Dr. Kuja-Panula | III |

**Table 3. Primary antibodies**

| Antigen | Description and clone | Source | Used in |
|---|---|---|---|
| Akt | Rabbit anti-mouse Akt | Cell Signaling Technology | III |
| α-Tubulin | Mouse anti-human α-Tubulin (clone B-7) | Santa Cruz Biotechnology | III |
| β-Actin | Mouse anti-mouse β-Actin (clone AC-15) | Sigma-Aldrich | III |
| CD11b | PerCP/Cy5.5-conjugated rat anti-mouse/human CD11b (clone M1/70) | BioLegend | II |
| CD11b/c | eFluor 660-conjugated mouse anti-rat CD11b/c (clone OX42) | eBioscience | I |
| CD172a | PE-conjugated mouse anti-rat CD172a (clone OX-41) | BioLegend | I |
| CD28 | Hamster anti-mouse CD28 (clone PV-1) | Beckman Coulter | III |

| | | | |
|---|---|---|---|
| CD206 | FITC-conjugated rat anti-mouse CD206 (clone C068C2) | BioLegend | II, III |
| CD3 | Hamster anti-mouse CD3 (clone 145-2C11) | Beckman Coulter | III |
| CD3 | FITC-conjugated Hamster anti-mouse CD3 (clone 145-2C11) | BioLegend | III |
| CD4 | PE/Cy7-conjugated anti-mouse CD4 (clone Gk1.5) | BioLegend | III |
| CD45 | PE-conjugated anti-mouse CD45 (clone 30-F11) | BioLegend | III |
| CD45 | APC-conjugated rat anti-mouse CD45 (clone 30-F11) | BioLegend | II, III |
| CD45 | PE-Texas Red-conjugated rat anti-mouse CD45 (clone I3/2.3) | Abcam | II |
| CD45R | PE/Cy5.5-conjugated anti-mouse CD45R (clone RA3-6B2) | BioLegend | III |
| CD69 | PE-conjugated anti-mouse CD69 (clone H1.2F3) | Beckman Coulter | III |
| CD8 | APC-conjugated anti-mouse CD8 (clone 53-6.7) | BioLegend | III |
| CD8 | PerCP/Cy5.5-conjugated anti-mouse CD8 (clone 53-6.7) | BioLegend | III |
| F4/80 | PE-Cy7-conjugated rat anti-mouse F4/80 (clone BM8) | BioLegend | II, III |
| GATA-3 | Mouse anti-mouse GATA-3 (clone C17.8) | BD Biosciences | I |
| Granulocyte | FITC-conjugated mouse anti-rat Granulocyte (clone HIS48) | eBioscience | I |
| Gr-1 | APC-conjugated rat anti-mouse Ly-6G/Ly-6C (Gr-1) (clone RB6-8C5) | BioLegend | II, III |
| GSK-3β | Rabbit anti-human GSK-3β | Santa Cruz Biotechnology | III |
| Iba-1 | Rabbit anti Iba-1 | Wako | I |
| IFN-γ | Hamster anti-mouse IFN-γ (clone XMG1.2) | BD Biosciences | III |
| IL-4 | Rat anti-mouse IL-4 (clone 11B11) | BD Biosciences | III |
| IL-12 | Rat anti-mouse IL-12 (clone 15.6) | BD Biosciences | III |
| IL-17A | Alexa Fluor 647-conjugated anti-IL-17A (clone TC11-18H10) | BD Biosciences | III |
| Lamin B | Goat anti-human Lamin B1 | Santa Cruz Biotechnology | III |
| MHCII | PE conjugated rat anti-mouse I-A/I-E (clone M5/114.15.2) | BioLegend | II, III |
| MHCII | PerCP-eFluor 710-conjugated mouse anti-rat MHCII (clone OX17) | eBioscience | I |
| NFAT1 | Mouse anti-mouse NFAT1 (clone 4G6-G5) | Santa Cruz Biotechnology | III |
| phospho-Akt (Ser473) | Rabbit anti-mouse phospho-Akt (Ser473) | Cell Signaling Technology | III |
| phospho-GSK-3β | Rabbit anti phospho-GSK-3α/β (Ser21/9) (clone D17D2) | Cell Signaling Technology | III |
| T-bet | Mouse anti-mouse T-bet (clone 4B10) | Santa Cruz Biotechnology | III |

**Table 4. Proteins**

| Protein | Description | Source | Used in |
|---|---|---|---|
| Avidin | A tetrameric biotin-binding protein | Merck Millipore | III |
| Concanavalin A (ConA) | A lectin protein acting as a mitogen | Sigma-Aldrich | III |
| IL-1β | Recombinant mouse IL-1β | R&D Systems | III |
| IL-4 | Recombinant mouse IL-4 | R&D Systems | III |
| IL-6 | Recombinant mouse IL-6 | R&D Systems | III |
| IL-12 | Recombinant mouse IL-12 | R&D Systems | III |
| TGF-β1 | Recombinant human TGF-β1 | R&D Systems | III |

**Table 5. Chemicals**

| Chemical | Source | Used in |
|---|---|---|
| CFSE | Thermo Fisher Scientific | III |
| eFluor 670 | eBioscience | III |
| Ionomycin | Sigma-Aldrich | III |
| Minocycline | Sigma-Aldrich | I |
| PMA | Sigma-Aldrich | III |
| Propidium iodide | Invitrogen | III |

## 4.2. Methods

| Methods | Used in |
|---|---|
| Active EAE induction | III |
| Cell culture | III |
| CNS mononuclear cell isolation by Percoll gradient centrifugation | II, III |
| Cytoplasmic and nuclear fractionation | III |
| Database for Annotation, Visualization and Integrated Discovery (DAVID) functional annotation clustering | I-III |
| Dual-luciferase reporter assay | III |
| EPM | II |
| ELISA | II |
| Flow cytometry | I-III |
| Immunohistochemistry (IHC) | I |
| Intracellular cytokine staining | III |
| Intraperitoneal injection | I-III |
| Intrathecal administration | I |
| Intravenous injection | III |
| Light-dark test (LD) | I, II |
| Microarray data analysis | I-III |
| Multiplex bead-based cytokine assay | III |
| Open field test | I, II |
| Polymerase chain reaction (PCR) | III |
| Pearson's co-efficiency analysis | II |
| Reverse transcription and RT-qPCR | I-III |
| RNA purification | I-III |
| T cell activation, proliferation and differentiation assay | III |
| T cell isolation | III |
| T cell homing assay | III |
| SNI surgery | I |
| Statistical analysis | I-III |
| Von Frey hair test | I |
| Western blotting and quantification by ImageJ | III |

The animals and main methods used are described below in details.

*Animals (I-III)*

Adult male Hannover-Wistar rats weighing 250-300 g, and 7-8-week-old male C57BL/6J, FVB/N, DBA/2J, and 129S2/Sv mice were purchased from Envigo (Horst, Netherlands). WT and AMG2KO C57BL/6J mice were routinely bred by heterozygous mating in a conventional or specific-pathogen-free (SPF) laboratory animal facility at the University of Helsinki (Helsinki, Finland). Rats were single-housed and mice were group-housed, whenever possible, in a 12 h light/dark cycle with food and water *ad libitum*. Experiment procedures were approved by the National Animal Experiment Board at the Regional State Administrative Agency for Southern Finland under licenses ESAVI/7863/04.10.07/2013 and ESAVI/706/04.10.07/2015.

*Anxiety-like behavioral tests (I-II)*

Animals were transported into an experimental room at least half an hour for adaptation prior to testing.

Elevated plus maze (EPM): Test was carried out in a plus maze consisted of a central zone, two open arms and two arms closed by transparent Plexiglas walls. The maze was elevated above the floor level. An animal was released to the center of the maze and monitored for 5 min. Video-tracking system was used to monitor travelled distance, number of entries and time spent in the closed and open arms.

Light-dark box (LD): Test was carried out in an experimental arena divided by a dark insert into two equal compartments. Dark and light zones were connected by an open arc to allow free movement between the compartments. An animal was released to the light zone and allowed to explore the experimental chamber for 10 min. Distance travelled, number of transitions between compartments, and time spent in each zone were detected by infrared sensors in an activity-monitoring system, and number of fecal boli excreted during the test were counted.

Open field (OF): Test was performed in a round chamber with transparent walls and white floor. An animal was placed to the arena facing the wall, and its activity was video-tracked for 5-30 min. Number of entries into and time spent in the central zone, as well as travelled distance, were detected by an activity-monitoring system.

*Chronic intrathecal minocycline treatment (I)*

Rats were implanted with intrathecal catheters (PE-10, BD) under general anesthesia with sodium pentobarbital (60 mg/kg body weight, i.p.). Starting from the operation day and 20 min before SNI surgery, one group received 50-μg intrathecal minocycline hydrochloride (Sigma-Aldrich) daily for two weeks whereas saline groups received the same volume of saline during the same period.

*Correlational analysis (II)*

Correlation between parameters was evaluated by Pearson's co-efficiency analysis.

*Dual-luciferase reporter assay (III)*

Full-length and cytosolic tail-truncated *Amigo2* cDNAs were cloned into signal-FLAG pIG mammalian expression vector. Mammalian expression plasmid, together with pGL3-NF-kB firefly luciferase reporter (50 ng) and pRL-TK reporter (10 ng), which serves as an internal control for transfection efficiency, were co-transfected into 293T cells in triplicates with Lipofectamine 2000 (Invitrogen). After 24 h, luciferase activities were measured by the Dual-Luciferase Assay System (Promega). Results are presented as relative activity of the NF-kB-driven firefly luciferase to that of the renilla luciferase.

*EAE induction (III)*

EAE was induced by immunizing 10-week-old female mice with subcutaneous injections of $MOG_{35-55}$ emulsified in complete Freund's adjuvant (CFA) at two sites, followed by intraperitoneally administration of two doses of pertussis toxin (400 ng per dose) dissolved in PBS on the same and the following day of immunization, using the Hooke Kit $MOG_{35-55}$/CFA Emulsion PTX (cat. No. EK-2110, Hooke Laboratories). Starting from 7 days post-immunization (dpi), mice were scored daily by at least two investigators for clinical signs of paralysis. The scoring criteria used were as follows: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 1.5, paralyzed tail and one hind limb paresis; 2, uncoordinated movement and hind limb paresis; 2.5, paralysis of one hind limb; 3, complete paralysis of both hind limbs; 3.5, complete paralysis of hind limb and weakness in forelimbs; 4, complete paralysis of both hind and forelimbs; and 5, moribound.

*Enrichment of T cells from murine spleen (III)*

Total T or Th cells were enriched from pooled spleens of adult mice using the Mouse EasySep T Cell Isolation Kit or the Mouse CD4+ T Cell Isolation Kit (Stem Cell Technologies). Naïve Th cells were enriched using the Mouse Naïve CD4+ T Cell Isolation Kit (Miltenyi Biotec).

*Flow cytometric analysis of immune cells from raw CNS tissues (I)*

Animals were anesthetized with sodium pentobarbital (Orion Pharma, Finland) and perfused intracardially with ice-cold PBS. Brain or spinal tissues were dissected, weighed, cut into tiny pieces and gently homogenized through 70-µm cell strainers (Fisher Scientific) in FACS buffer (PBS supplemented with 1% heat-inactivated FBS and 0.02% $NaN_3$). Single cell suspensions prepared from ~25 mg tissue per sample were blocked with 5% normal rat serum, and stained with a combination of flow markers (Granulocyte-FITC, CD172a-PE, MHCII-PerCP-eFluor 710, and CD11b/c-eFluor 660) with light protection at 4°C under a 60-min continuous rotation.

Specificities of flow markers were confirmed by comparing with respective isotype controls. After washing, cells were resuspended into 2 ml FACS buffer, and acquired on a 2-laser, 6-color Gallios cytometer (Beckman Coulter) under a live gate of CD11b/c$^+$. Flow cytometric data were analyzed with the Kaluza 1.3 analysis software (Beckman Coulter).

*Flow cytometric analysis of immune cells prepared from immune organs or enriched from CNS tissues (II-III)*

Cells were blocked with 10% normal rat serum in PBS on ice for 30 min, followed by staining with anti-mouse flow markers with light protection on ice for another 30 min. After staining, cells were washed and resuspended into 500 µl FACS buffer. Samples were acquired with a 2-laser, 6-color Gallios cytometer and flow cytometric data were analyzed with the Kaluza 1.3 analysis software.

*Immunohistochemistry (I)*

Animals were anesthetized with sodium pentobarbital and perfused intracardially with PBS followed by 4% paraformaldehyde (PFA) in PBS. Lumbar (L4-6) SCs were dissected and post-fixed in 4% PFA at 4°C for overnight. After post-fixation, the SCs were transferred to 30% sucrose in PBS for two days of dehydration, and finally frozen in Tissuetek (Sakura) and cut into 20-µm floating sections with a cryostat (Leica). Cryosections were blocked with 5% normal goat serum and permeabilized with TBST (TBS supplemented with 0.1% Tween-20) and incubated with a rabbit anti-Iba1 polyclonal antibody (Wako Chemical, Japan), followed by an Alexa Fluor 568-conjugated goat anti-rabbit IgG antibody (Life Technologies). Stained sections were mounted onto glass slides and imaged by a Zeiss Axioplan 2 microscope under the 10× magnitude and an Axiocam HR camera (Carl Zeiss).

*In vitro Th cell activation, proliferation and differentiation assays (III)*

In Th cell activation assay, anti-CD3 with or without anti-CD28 antibody was pre-coated into 48-well plates (Corning) in 150 µl PBS per well at 4°C for overnight. Splenic Th cells ($1 \times 10^6$) resuspended in 0.4 ml complete RPMI 1640 medium (Lonza) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 10 mM HEPES, 1 × NEAA (Gibco), and 1 mM sodium pyruvate were then applied into the wells and cultured at 37°C in a humidified cell incubator with 5% $CO_2$. After 24 h, the cells were collected, washed, blocked with 10% normal rat serum, and stained with a combination of anti-CD69-PE, CD4-PE/Cy7, and CD45-APC antibodies on ice with light protection for 30 min before flow cytometry. T cell activation index is presented as the percentage of CD69$^+$ Th cells among total Th cells.

In Th cell proliferation assay, splenic Th cells were fluorescently labeled with CFSE and then stimulated with different concentrations of anti-CD3 antibody or ConA in 24-well plates at 37°C

in a humidified cell incubator with 5% $CO_2$. After 72 h, the cells were collected, blocked with 10% normal rat serum and stained with anti-CD4-APC antibody on ice with light protection for 30 min. Following washing, the cells were resuspended into FACS buffer and propidium iodide (PI; 1:500) was added prior to flow cytometry. T cell proliferation index is presented as the percentage of $CFSE^+$ dividing cells among total $PI^-CFSE^+$ live Th cells.

In Th cell differentiation assay, naïve splenic Th cells were stimulated in 24-well plates pre-coated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) antibodies in combination with the following polarizing cytokines and neutralizing antibodies: Th0, 10 μg/ml anti-IFN-γ, 10 μg/ml anti-IL-4, and 10 μg/ml anti-IL-12; Th1, 30 ng/ml IL-12 and 10 μg/ml anti-IL-4; Th2, 30 ng/ml IL-4,10 μg/mL anti-IL-12 and 10 μg/mL anti-IFN-γ; and Th17, 1 ng/ml TGF-β1, 10 ng/ml IL-1β, 20 ng/ml IL-6, 10 μg/ml anti-IFN-γ and 10 μg/ml anti-IL-4. Cells were cultured in Iscove's modified Dulbecco medium (Sigma) supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, 1 × NEAA, 0.05 mM 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified cell incubator with 5% $CO_2$. After the time indicated, cells were collected and prepared for Western blotting (Th0, Th1, and Th2) or intracellular cytokine staining with anti-IL-17A-Alexa Fluor 647 (Th17) for flow cytometry. Cytokine levels in culture supernatants were measured using the bead-based Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Kit (eBioscience) with flow cytometry.

*In vivo T cell homing assay (III)*

Total T cells enriched from the spleens of adult WT and AMG2KO mice were fluorescently labeled with CFSE and eFluor 670, respectively. The fluorescently labeled donor T cells were washed and mixed (1:1), and approximately $9 \times 10^6$ cells were injected intravenously into tail veins of WT recipient mice. After 15 h, donor T cells that accumulated into secondary lymphoid organs including spleen, lymph nodes, and Peyer's patches of the recipient mice were assessed by staining with anti-CD45-PE, CD8-PerCP/Cy5.5 and CD4-PE/Cy7 antibodies with flow cytometry.

*Isolation of CNS leukocytes, and bone marrow cells, splenocytes and thymocytes (II-III)*

Animals were anesthetized with sodium pentobarbital or euthanized with $CO_2$. Spleens were quickly dissected before intracardial perfusion was done with ice-cold PBS. Brains or SCs were dissected, cut into small pieces and gently homogenized through 70-μm cell strainers (Fisher Scientific) with plungers from 2-ml syringes in 7 ml RPMI 1640 medium supplemented with 2 mM EDTA (pH 7.0). Afterward, 3 ml of 100% isotonic Percoll (GE Healthcare) were added into the brain or spinal homogenates and mixed thoroughly to make a final 30% isotonic Percoll, which was carefully layered on top of 70% isotonic Percoll. The gradient was then centrifuged at

500 × g for 30 min without brake. Leukocytes were collected from the interphase between Percoll layers and washed with ice-cold PBS.

Spleens and thymuses were gently grinded through 40-µm cell strainers (Fisher Scientific) with plungers of 2-ml syringes, femoral and tibial bone marrow were flushed out by using 25-gauge needles attached to 1-ml syringes filled with PBS. The splenocytes and bone marrow cells were erythro-lyzed with the VersaLyse Lysing Solution (Beckman Coulter), and washed with ice-cold PBS.

*Leukocyte quantification in the CNS of EAE mice (III)*

At 9 dpi, mice were euthanized with $CO_2$, perfused with ice-cold PBS, brains and SCs were carefully dissected, and mononuclear cells from these CNS tissues were enriched as described previously (Li et al., 2014). Enriched cells were blocked with 10% normal rat serum and stained with a combination of the following flow markers: CD3-FITC, CD4-PE/Cy7, and CD45-APC, or CD206-FITC, MHCII-PE, F4/80-PE/Cy7, and CD45-APC on ice with light protection for 30 min for flow cytometry.

*LPS treatment (II)*

In LPS groups, each mouse was injected intraperitoneally with 1 mg/kg body weight of *Escherichia coli* O111:B4 LPS (Sigma-Aldrich) in PBS. Control groups received injection with the same volume of PBS. Naïve groups did not receive any treatment.

*$MOG_{35-55}$-restimulated splenocytic recall response in vitro and multiplex bead-based cytokine assay (III)*

At 14 dpi, mice were euthanized with $CO_2$ and spleens were dissected. Spleens were gently homogenized through 70-µm cell strainers in RPMI 1640 medium supplemented with 2% heated-inactivated FBS and 10 mM HEPES. Splenocytes were erythro-lyzed with VersaLyse Lysing Solution, washed and filtered through 70-µm cell strainers again. Two million splenocytes were re-stimulated with 20 µg/ml $MOG_{35-55}$ peptide (Hooke Laboratories) in 48-well plates at 37°C in a humidified cell incubator with 5% $CO_2$. After 72 h, culture supernatants were collected and cytokine levels were measured using the bead-based Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Kit with flow cytometry.

*Neuropathic pain-like behavioral test (I)*

Animals were habituated to an experimental room one hour per day for three days. Mechanical sensitivity was assessed by a calibrated series of von Frey monofilaments producing forces ranging from 0.4 to 60 g (0.4, 1, 2, 4, 6, 8, 10, 15, 26, and 60 g, North Coast Medical Inc). During the test, an animal was placed on a grid and allowed to move freely inside a transparent box. The monofilaments below the grid were applied to a hind paw of the animal with increasing forces

until the animal withdrew it. Ipsilateral hind paw was stimulated for five times at each stimulus force with an ascending series of the monofilaments. At each stimulus force, number of withdrawal responses was counted, and withdrawal response rate (%) was calculated. An increase of the withdrawal response rate was considered to represent neuropathic pain-like mechanical hypersensitivity.

*SNI surgery (I)*

To induce neuropathic pain in rats, SNI model was adopted (Decosterd and Woolf, 2000). Before SNI surgery, a rat was anesthetized with 60 mg/kg body weight of sodium pentobarbital intraperitoneally. An incision was subsequently made into the skin on the lateral surface of left thigh, followed by a section through biceps femoris muscle to expose the sciatic nerve and its terminal branches: sural, common peroneal and tibial nerves. The common peroneal and tibial nerves were then tightly ligated with 4-0 silk and sectioned at sites distal to the ligation. Approximately 3-4 mm of the distal nerve stumps were then removed. The sural nerve was left intact without stretching. For the sham-operated rats, the sciatic nerve was exposed in the same manner but without ligation. To prevent post-operative pain, animals were treated with 0.01 mg/kg body weight of buprenorphine twice daily for three days.

*Total RNA purification and RT-qPCR (I-III)*

Total RNA from tissues or cells was extracted using the GeneJET RNA Purification Kit (Thermo Scientific), treated with the TURBO DNA-free DNase (Ambion) to remove trace amount of genomic DNA, and reversely transcribed (200 ng) with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). RT-qPCR was performed by using corresponding primers and the Maxima SYBR Green qPCR Master Mix (Thermo Scientific) on the CFX384 Real-Time PCR Detection System (Bio-Rad) according to manufacturer's instructions. Quantification was performed by normalization with house-keeping gene *Actb* or *Gapdh*, and represented as fold change $2^{-\Delta\Delta Ct}$.

*Th cell stimulation and subcellular fractionations (III)*

Avidin (20 µg/well) was coated into 24-well plates at 4°C for overnight. After blocking with 1% bovine serum albumin (BSA) in PBS, biotinylated anti-CD3 (315 ng/well) and anti-CD28 (200 ng/well) antibodies were applied to the wells and incubated at room temperature for at least 3 h. Splenic Th cells ($4\sim5 \times 10^6$) resuspended in 0.5 ml of complete RPMI 1640 medium were then added and stimulated at 37°C in a humidified cell incubator with 5% $CO_2$ for 0, 1, and 3 h before cell lysis. For subcellular fractionation, splenic Th cells were firstly rested in complete RPMI 1640 medium at 37°C for 1 h, and stimulated with PMA (50 ng/ml, Sigma-Aldrich) and ionomycin (Iono, 1 µg/ml, Sigma-Aldrich) at 37°C for 10 min. The cells were then pelleted and

washed once with ice-cold PBS. Cytoplasmic and nuclear fractions from stimulated Th cells were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific).

*Western blotting (III)*

Cells were pelleted, lysed with Laemmli lysis buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 25 mM Tris-HCl and 0.002% bromophenol blue) and boiled at 100°C for 10 min. The lysates were then separated on 4-15% gradient SDS-PAGE gels and transferred onto nitrocellulose membranes using the Trans-blot Turbo Transfer System (Bio-Rad). Blots were blocked with 5% non-fat milk or BSA in TBST (TBS supplemented with 0.1% Tween-20) at room temperature for one hour, followed by overnight incubation at 4°C with respective primary antibodies. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for one hour, and finally developed with ECL (Thermo Scientific) or ECL Plus (GE Healthcare) substrates. The developed membranes were imaged by G:Box Chemi XX6 imager (Syngene). Densitometry analysis of blotting images was done by using the quantificational method in ImageJ.

*Statistics (I-III)*

Data were analyzed by one- or two-way analysis of variance (ANOVA) with Bonferroni's post hoc or Fisher's LSD test. Two-group comparison was performed with Student's *t* test (for parametric data) or Mann-Whitney U test (for nonparametric data). All values presented were means ± standard errors of mean (SEM) or standard deviations (SD). Statistical significance was set to $p < 0.05$. *P* values were classified as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

# 5. Results and discussion

## 5.1. Role of microglia/macrophages in neuropathic pain (I)

### 5.1.1. Elevated number of microglia/macrophages in the lumbar spinal cord (SC) of SNI rats

Sciatic nerve injuries are the most commonly used rodent experimental models for neuropathic pain. To explore temporal-spatial roles that microglia/macrophages play in the CNS (the brain and the SC) during the course of neuropathic pain, we chose to adopt the widely used SNI model in rats. After surgical procedures, we first validated the development of neuropathic pain-like mechanical hypersensitivity by the classic Von Frey hair test and comorbid anxiety-like behavior by LD test in rats at different stages (post-operative day (POD)3, 6, and 20) following SNI surgery (**I, Fig. 1**).

Among experimental approaches to study microglial responses, IHC is the most popular one as it retains not only morphological but also anatomic information. By utilizing IHC approaches, several previous studies revealed the occurrence of early microgliosis and microglial activation (upregulation of CD11b/c) in the SDH following a peripheral nerve injury (Blackbeard et al., 2007; Echeverry et al., 2008). In our study, we also used IHC methods to assess the density of spinal microglia/macrophages by staining with the microglia/macrophage-specific antibody Iba-1. In line with other reports, we found large focal increases of microglia/macrophages in the ipsilateral spinal dorsal and ventral horns of SNI rats on both POD7 and POD21 (**I, Fig. 1**).

However, we should bear in mind that IHC has some disadvantages as well, such as limited availability of IHC antibodies and restrained capability for multiplex antibody staining on the same cell, and is a subjective, semi-quantitative analytical method. In this respect, flow cytometry has overcome the majority of these limitations and emerged as a convenient and robust technique with high sensitivity, efficiency, and reproducibility. Hence, we also employed a flow cytometry-based approach to measure total numbers of microglia/macrophages and to characterize their inflammatory polarization states in the brain and SC. So far, the majority of previously described methods involve an enrichment step of CNS tissues with Percoll gradient centrifugation prior to analysis (Cardona et al., 2006; Li et al., 2014; Pino and Cardona, 2011). However, due to limited availability of tissues in a specific brain or spinal region, we developed an approach to analyze microglia/macrophages in small amounts of raw CNS tissue samples. After blocking with normal rat serum, single cell suspensions prepared from raw CNS tissues were stained with a

combination of anti-rat cell surface flow markers (CD11b/c, Granulocyte marker, MHCII, and CD172a). Using a live gate of CD11b/c$^+$, we were able to measure the total number of microglia/macrophages (CD11b/c$^+$Granulocyte$^-$ cells) and to characterize their inflammatory polarization states (MHCII$^+$ and CD172a$^+$ microglia/macrophages) in a small amount (~25 mg) of raw CNS tissue, such as the lumbar SC and the PFC (**I, Figs. 2 and 3**).

By comparing the total number of microglia/macrophages (normalized to tissue weight) in the lumbar SC at different stages (POD3, 7 and 21) following SNI surgery, we found that more microglia/macrophages were present in the ipsilateral lumbar SC on both POD7 and POD21. In addition, we also noted upregulated CD11b/c expression by microglia/macrophages on POD21 (**I, Fig. 2**). In agreement with previous findings using IHC, our flow cytometric data demonstrated the validity of our flow cytometry-based approach and that microgliosis taking place at an early stage persists for at least three weeks following a peripheral nerve injury (**I, Fig. 2**).

### 5.1.2. Microglia/macrophages in the lumbar SC and PFC show opposite inflammatory responses to SNI

To characterize inflammatory responses that microglia/macrophages may exert within the CNS during the course of neuropathic pain, we compared the inflammatory polarization states of microglia/macrophages at different stages following SNI surgery. Due to limited availability of flow markers for rats, we only used two flow markers (MHCII and CD172a) to define pro-inflammatory M1 and anti-inflammatory M2 microglia/macrophages, respectively. We nevertheless considered MHCII$^+$ microglia/macrophages more pro-inflammatory and CD172a$^+$ cells more anti-inflammatory based on the known immune functions that MHCII and CD172a may be involved in (Linnartz and Neumann, 2013; Taylor et al., 2005).

When comparing microglial/macrophage inflammatory profiles (MHCII$^+$ and CD172a$^+$ subtypes) in the lumbar SC at different stages (POD3, 7 and 21) following SNI surgery, we did not observe any differences between sham- and SNI-operated rats regarding the percentages of MHCII$^+$ or CD172a$^+$ microglia/macrophages in the early stages (POD3 and 7). However, at a late stage (POD21), microglia/macrophages in the lumbar SC exhibited an anti-inflammatory response, represented by a decreased MHCII$^+$/CD172a$^+$ microglia/macrophage ratio (regarded as an inflammatory index indicating the balance between pro- and anti-inflammatory microglial/macrophage subtypes) (**I, Fig. 2**). Further quantitative analysis revealed that most of the proliferating microglia/macrophages in the lumbar SC were in fact CD172a$^+$

microglia/macrophages (**I, Fig. S1**). In contrast to a pro-inflammatory role as speculated in the literatures, according to our observations the spinal microglia/macrophages exhibited an anti-inflammatory and phagocytic phenotype in neuropathic pain. This is reasonable, considering the fact that microglia are guardians of the CNS and attempt to perform a neuroprotective function within the CNS in case of disease or injury. However, sufficient time may be required for microglia to achieve this goal.

The PFC is one of supraspinal brain regions that are sensitized by ascending signals from SDH in neuropathic pain. In addition to the hippocampus and amygdala, PFC is also a critical brain region that is closely associated with anxiety. Because SNI rats were more anxious (**I, Fig. 1**), we also analyzed the inflammatory profiles of microglia/macrophage in the PFC. Similar to the lumbar SC, we did not find any changes in $MHCII^{+}/CD172a^{+}$ ratio of microglia/macrophage in the early stages following SNI (POD3 and 7). However, at a later stage (POD21) and in contrast to the anti-inflammatory response in the lumbar SC, microglia/macrophages in the PFC exhibited a strikingly different pro-inflammatory response, as represented by lower percentages of $CD172a^{+}$ microglia/macrophages in the PFCs of SNI rats as compared to sham-treated rats (**I, Fig. 3**).

To overcome the limitation of flow cytometry, we employed RT-qPCR methods to compare mRNA expression of multiple microglia/macrophage-related genes in the ipsilateral SC, ipsilateral DRG, and contralateral PFC of both SNI and sham-treated rats. These genes included the microglial/macrophage activation marker *Cd68*, the major inhibitory Fcγ receptor (*Fcgr*)*2b*, the phagocytic receptor mannose receptor, C type 1 (*Mrc1*), complement genes (*C1qb*, *C3*, and *C4a*), pro- versus anti-inflammatory cytokines (*Il1b*, *Il10*, *Tnf*, and *Tgfb1*), and the M1/M2 microglia/macrophage signature genes (inducible nitric oxide synthase (*Nos2*) and Arginase (*Arg*)*1*). RT-qPCR analysis further corroborated our flow cytometric data and demonstrated that spinal and cortical microglia/macrophages exhibited opposite inflammatory responses to SNI. Namely, the lumbar spinal microglia/macrophages showed an anti-inflammatory and phagocytic response, whereas their prefrontal cortical counterparts showed a dampened capacity to exert anti-inflammatory and phagocytic functions following SNI surgery (**I, Fig. 4**).

Based on our observations, instead of a homogenous response to SNI, microglia/macrophages in the SC and PFC showed unexpected CNS region-specific inflammatory responses to a peripheral nerve injury.

### 5.1.3. The central nervous system (CNS) region-specific immune profiles in naïve rats

As the finding that microglia/macrophages in the SC and PFC exhibit opposite inflammatory responses at a late stage following a peripheral nerve injury was intriguing, we decided to investigate possible mechanisms. We first considered the possibility that microglia/macrophages might be different in different CNS regions under steady-state conditions. To test our hypothesis, we compared microglial/macrophage profiles (abundancy, pro- and anti-inflammatory polarization states, and cell surface expression of immune markers) in the lumbar SC, thalamus (as a representative sub-cortical brain region) and cortex of naïve rats by flow cytometry (**I, Fig. 3 and S2**). Regarding abundancy, we found that microglia/macrophages were most abundantly distributed in the cortex and least in the lumbar SC, with thalamus falling in between. When comparing their pro- versus anti-inflammatory subtypes, we found that the lumbar SC had the highest percentage of $MHCII^+$ microglia/macrophages and lowest percentage of $CD172a^+$ microglia/macrophages, whereas these parameters were completely reversed in the brain cortex. With regard to cell surface expression of immune markers, the lumbar SC expressed higher levels of CD11b/c and MHCII than the brain cortex. Collectively, these data support our hypothesis that microglial/macrophage immune profile is CNS region-specific in physiological conditions.

In addition, we also performed bioinformatics analysis based on published transcriptomic datasets of different brain regions or between the brain and the SC. Interestingly, we found that several clusters of immune genes were differentially expressed among different CNS regions, which provided further support of our hypothesis that basal immune profile of microglia/macrophages is CNS region-specific (**I, Table S2**). The underlying mechanism for the differential immune states of brain and spinal microglia/macrophages is currently unclear. Based on the literatures, we speculated that intrinsic programing of microglial/macrophage differentiation during CNS development is region-specific, due to the residing microenvironment provided by neural progenitor cells, neurons, or other cell types. For instance, neuronal CD200, an important ligand for microglia receptor CD200R, was expressed at different levels within the brain (Olson, 2010). Moreover, differences in the permeability of the blood-brain-barrier versus blood-SC barrier were observed, due to, for example, the anatomical differences in vascular structure (Schnell et al., 1999), which may also be a contributing factor.

### 5.1.4. Microglial/macrophage inhibitor minocycline attenuates pain hypersensitivity in early stages following SNI

Minocycline is a drug that efficiently dampens M1-type of microglia/macrophages. It has emerged as a potential drug candidate for treating neuropathic pain. However, preclinical studies on the analgesic effect of minocycline have so far given mixed results. Some studies reported that minocycline was able to prevent the development of but unable to reverse existing mechanical allodynia (Chang and Waxman, 2010; Willemen et al., 2010). Others have shown that prophylactic minocycline treatment failed to alleviate pain hypersensitivity (Burke et al., 2014; Taylor et al., 2015). To understand the role of CNS region-specific microglial/macrophage responses in the course of neuropathic pain, we utilized minocycline to modulate microglial/macrophage polarization in the SC to understand the role they play in chronic neuropathic pain.

In preclinical studies of neuropathic pain, both systemic and local minocycline treatment regimens have been used. Considering the therapeutic relevance in clinical treatment, we first applied minocycline systemically by intraperitoneal injections into SNI rats starting from the 4$^{th}$ day following SNI surgery for a period of two weeks. Although minocycline was able to dampen pro-inflammatory activation of spinal microglia/macrophages, this treatment regimen failed to alleviate neuropathic pain-like mechanical allodynia (**I, Fig. S4**). Due to this failure, we then evaluated the efficacy of local and prophylactic minocycline treatment on mechanical allodynia and microglial/macrophage activation. Interestingly, this treatment regimen alleviated mechanical allodynia efficiently until POD10 but not afterwards, with the anti-allodynic effect being the strongest during POD1-3 (**I, Fig. 5**). In line with previous reports (Ledeboer et al., 2005; Raghavendra et al., 2003; Sung et al., 2012; Yamamoto et al., 2015), our data demonstrated the efficacy and inefficacy of minocycline in treating neuropathic pain by different regimens.

When characterizing microglial/macrophage immune responses in the lumbar SC, we found that chronic intrathecal minocycline treatment efficiently dampened pro-inflammatory activation of spinal microglia/macrophages at both early (POD3) and late (POD13) stages (**I, Fig. 5**). In contrast, although minocycline treatment was able to downregulate CD11b/c expression on microglia/macrophages in the brain (the PFC and primary somatosensory cortex) on POD3 and POD13 (**I, Fig. S5**), it failed to dampen pro-inflammatory activation of microglia/macrophages (data not shown).

Our observations show that although intrathecal minocycline treatment exerts immediate efficacy on alleviating neuropathic pain-like mechanical hypersensitivity, possibly due to promoting anti-inflammatory responses of spinal microglia/macrophages, this effect is not long-lasting despite its ongoing anti-inflammatory effect on spinal microglia/macrophages at later stages. We speculate that the inefficacy of minocycline at late stages may be due to the unresolved brain inflammation at chronic stages of neuropathic pain.

In summary, we discovered that microglia/macrophages in the CNS are region-specific, in terms of their abundancy, inflammatory polarization states, and expression of multiple microglia/macrophage-related immune molecules in the physiological condition. Such CNS specificity of microglia/macrophages may underlie their subsequent opposite inflammatory responses to SNI. In addition, our data provide a possible explanation of the controversial efficacy (and inefficacy) of minocycline in attenuating neuropathic pain-like hypersensitivity and suggest that future treatment strategies targeting both spinal and brain inflammation may be more powerful in alleviating neuropathic pain in chronic stages. Of course this speculation warrants further studies in the near future.

## 5.2. Role of microglial polarization in anxiety disorders (I&II)

### 5.2.1. Anxiety as a comorbid behavior with neuropathic pain in rats

Patients with neuropathic pain frequently develop comorbidities, such as anxiety, depression, and sleep disturbance (Alexander et al., 2014). Thus, we performed LD tests to assess anxiety-like behaviors for both SNI and sham-operated rats on POD0, 6, and 20. Compared to sham-operated controls, SNI rats secreted significantly larger numbers of fecal boil in the LD box within POD20 (**I, Fig. 1**), suggesting a time-dependent development of anxiety-like behavior in these rats.

### 5.2.2. Inbred mouse strains with differential anxiety traits have different microglial and macrophage subpopulations

Microglia can be triggered by not only psychosocial stressors, but also peripheral immune challenge, such as pathogenic infection. Microglia also produce pro-inflammatory cytokines, which modulate animal's behaviors. In terms of immune functions, microglia can be polarized to either a pro-inflammatory M1 or anti-inflammatory M2 state. However, evidence on the

association of microglial pro- versus anti-inflammatory polarization with anxiety is currently lacking.

Due to rich genetic, physiological, and behavioral information of inbred mouse strains in public databases, we decided to use inbred mouse strains with differential anxiety traits in our studies. To choose mouse strains, we first explored Mouse Phenome Database and identified C57BL/6J, FVB/N, DBA/2J and 129S2/Sv as appropriate strains for such purposes, based on the fact that DBA/2J and 129S2/Sv male mice are more anxious than their C57BL/6J and FVB/N male counterparts. In our hands, we further validated their anxiety-like behaviors using classical anxiety behavioral tests including EPM, LD, and OF (**II, Fig. 1**).

In flow cytometry, CD45 can be used as a marker to distinguish microglia from peripheral macrophages. In this study, to decipher microglial pro- versus anti-inflammatory activation, we used a combination of anti-mouse cell surface flow markers including CD206-FITC, MHCII-PE, CD11b-PE/Cy5.5, F4/80-PE/Cy7, and CD45-APC. This allowed us to specifically distinguish microglia ($CD45^{lo}F4/80^{+}$ cells) from peripheral macrophages ($CD45^{hi}F4/80^{+}$ cells) and to analyze microglial subpopulations (pro-inflammatory $MHCII^{+}CD206^{-}$ M1 and anti-inflammatory $MHCII^{-/+}CD206^{+}$ M2 microglia) in the brain (**II, Fig. 2**).

Since these inbred mouse strains exhibit differential anxiety traits, we next asked whether microglial and macrophage profiles in their brains would be different under steady-state conditions. Our results revealed that although microglia did not differ in total numbers in the brains of these mouse strains, microglial M1/M2 ratio in a high-anxiety strain (DBA/2J) was higher than the other strains. Moreover, another high-anxiety mouse strain (129S2/Sv) had a higher $MHCII^{+}/MHCII^{-}$ macrophage ratio than the other strains (**II, Fig. 2**). Collectively, these data suggest that microglia and macrophages in the brain are different between high- and low-anxiety mouse strains and that myeloid cellular constituents may be different even among high-anxiety strains.

### 5.2.3. Microglia are more M1-polarized in high-anxiety mouse strains after a systemic lipopolysaccharide (LPS) challenge

Based on the observation that brain microglia and macrophages showed some differences under steady-state conditions, we then subjected these inbred mouse strains to an acute intraperitoneal LPS challenge and examined brain microglial profiles after 15 h. As expected, all mouse strains had increased M1/M2 microglial ratios in their brains in response to LPS challenge. Interestingly,

the increase of M1/M2 microglial ratio was more pronounced in the high-anxiety strains (DBA/2J and 129S2/Sv) than in the low-anxiety strains (C57BL/6J and FVB/N) (**II, Fig. 4**).

To examine whether LPS challenge can induce similar changes of macrophages in the peripheral immune organs, we compared splenic macrophages in these four inbred mouse strains. We found that the high-anxiety strains (DBA/2J and 129S2/Sv) had more pronounced increases of the M1/M2 macrophage ratio than low-anxiety strains (C57BL/6J and FVB/N) (**II, Fig. 5**), which is in line with microglial responses in the brain (**II, Fig. 4**). These data demonstrate that brain microglia and splenic macrophages exhibit synergized responses to peripheral LPS challenge and further suggest that M1/M2 microglial ratio in the brain may reflect anxiety trait of a mouse strain.

### 5.2.4. High-anxiety inbred mouse strains express higher levels of pro-inflammatory cytokines and M1 microglia signature genes in the hypothalamus

To corroborate our flow cytometric data, we further measured mRNA levels of pro-inflammatory genes, including *Il1b*, *Il6*, and *Tnf*, in the hypothalamus of phosphate-buffered saline (PBS)-treated control mice, and microglial M1/M2 signature genes (*Nos2* and *Arg1*) in the hypothalamus of LPS-treated mice using RT-qPCR. The hypothalamus is a brain region sensitive to both acute stress and peripheral LPS stimulation. We chose to measure cytokine levels in PBS-injected control mice, because cytokine levels in naïve brains were barely detectable whereas their expression might be induced by a mild and transient handling stress in PBS groups. Compared with the low-anxiety strains (C57BL/6J and FVB/N), RT-qPCR analysis revealed that the high-anxiety strains (DBA/2J and 129S2/Sv) expressed higher levels of *Il1b* and *Tnf* in the hypothalamus. More interestingly, when comparing the mRNA levels of microglial M1 and M2 signature genes in the hypothalamus of LPS-treated mice, we found that *Nos2*/*Arg1* ratios were also higher in the high-anxiety strains (DBA/2J and 129S2/Sv) than the low-anxiety strains (C57BL/6J and FVB/N) (**II, Fig. 6**). These data provide further support that brain microglia in high-anxiety mouse strains are more polarized to pro-inflammatory M1 type.

### 5.2.5. M1/M2 microglial ratios are correlated with anxiety-like behaviors

Based on the above knowledge, we then performed Pearson's correlational analysis to see whether M1/M2 microglial ratios in the mouse brains after a peripheral LPS challenge were associated with anxiety-like behaviors in inbred mice. Interestingly, the M1/M2 microglial ratio was negatively correlated with the number of light zone entries in the LD test and the percentage

of distance traveled in the center in the OF test (**II, Fig. 7**), demonstrating that the more anxious an animal is, the higher M1/M2 microglial ratio it has in the brain.

### 5.2.6. Putative myeloid transcription factors and behavioral genes underlying differential microglial polarization profiles across inbred mouse strains

Although both DBA/2J and 129S2/Sv are considered high-anxiety mouse strains, their innate immune profiles in the brain were different according to our analysis. We speculated that in addition to canonical pro-inflammatory cytokines, other immune mechanisms might also contribute to differences in the microglial activation profile. In fact, by comparing the public whole brain microarray data in PhenoGen Informatics (Bhave et al., 2007), we found that 129S2/Sv mice expressed higher levels of multiple immune genes, such as *Ccl8*, *Cd24a*, *Fcgr2b*, *Il12a*, and *Tlrs* (**II, Table 2**).

To better understand the underlying genetic mechanisms of anxiety-associated microglial activation, we performed transcriptomic profiling analysis to identify genes ($r \geq +0.95$ or $\leq -0.95$ and $p < 0.05$) that were most significantly correlated with M1/M2 microglial ratios. These candidate genes were further imported into DAVID for functional annotation clustering. To our interest, we discovered a group of positively correlated transcription factors that are involved in hematopoietic development (**II, Table 3**). Moreover, we also found a group of behavioral genes that were negatively correlated with brain M1/M2 microglial ratios. These genes may also underlie differential microglial profiles among these inbred mouse strains.

In summary, using a variety of experimental approaches, we discovered for the first time a correlation between brain microglial M1/M2 ratio and anxiety-like behavior in inbred mice, implying microglial M1/M2 ratio in the brain as a potential biological index of anxiety in mice or a useful readout when screening potential therapeutic drugs for treating anxiety disorders.

## 5.3. AMIGO2 in regulation of immune cell functions and in EAE (III)

### 5.3.1. Implication of AMIGO2 in the regulation of T-cell function and in EAE

In the *Amigo* gene family, *Amigo1* is enriched in the CNS of adult mouse whereas *Amigo2* and *Amigo3* are more widely distributed across tissues (Kuja-Panula et al., 2003). Consequently, most

studies concerning AMIGO1 have focused on its involvement in the regulation of neuronal function. Concerning AMIGO2, the situation is rather similar. Nevertheless, scanty yet accumulating evidence indicates that AMIGO2 may play a role in regulating T-cell function (Lund et al., 2007; Tsukumo et al., 2006).

To identify immune cell population or tissue type in which *Amigo2* is abundantly expressed, we first explored two online microarray databases (Immgen and IST Online) for gene expression patterns of *Amigo1~3* in various murine and human tissue and cell types. Interestingly, *Amigo2* was most abundantly expressed in murine thymic $CD4^+$ single-positive Th cells. However, we did not observe such a differential expression pattern of *Amigo1* and *Amigo3* in T cells, suggesting a potential role of AMIGO2 in regulation of T-cell function (**III, Fig. S1**). Similarly, *AMIGO2* was most abundantly expressed in lymphocytes in the human tissue and cell types (**III, Fig. S2**). To validate such a differential expression pattern, we performed RT-qPCR analysis using total RNA purified from adult WT murine splenic Th cells and confirmed that *Amigo2* was the most abundantly expressed *Amigo* gene in murine Th cells (**III, Fig. 1**).

Since EAE is mainly a Th1- or Th17-driven autoimmune disease model, we next asked whether EAE might induce differential *Amigo2* expression in Th cells. For this purpose, we induced acute EAE in WT female C57BL/6J mice, and enriched splenic Th cells at 14 dpi for RT-qPCR analysis. *Amigo2* mRNA was dramatically downregulated in EAE-sensitized splenic Th cells (**III, Fig. 1**), indicating that AMIGO2 may be critically involved in EAE pathogenesis and its expression in Th cells may be subtype specific. Collectively, these data suggest a potential involvement of AMIGO2 in regulation of T-cell function and in acute EAE.

To explore roles of AMIGO2 *in vivo*, we generated a conventional AMG2KO mouse line on the C57BL/6J background by replacing the full-length *Amigo2* coding sequence with the human placental alkaline phosphatase gene. The inactivation of *Amigo2* was confirmed by both PCR genotyping and RT-PCR with total RNA extracted from the murine spleen and thymus (**III, Fig. 1**). Under both conventional and SPF housing environments, these mice developed normally without any obvious defects. We then asked whether *Amigo2*-deficiency might affect T cell development and maturation. Thus, we immunophenotyped T cells in the thymus and spleen of mice housed in both conventional and SPF environment and found a slight increase of T cells in the spleens of AMG2KO mice housed in conventional environment (**III, Fig. 2**).

Since AMIGO proteins share a high similarity at the amino acid level (50-55%), we then assessed whether *Amigo2*-deficiency in mice would result in any compensational expression of other *Amigo*s, thereby masking the functionalities of AMIGO2. For this purpose, we measured mRNA levels of *Amigo*s in the splenic Th cells by RT-qPCR. *Amigo1* and *Amigo3* mRNA expression did not change in the splenic Th cells of AMG2KO mice compared to WT (**III, Fig. 1**), suggesting no compensational expression (and probably no functional compensation) from other AMIGOs in the splenic Th cells of AMG2KO mice.

### 5.3.2. AMIGO2 functions as an adhesion molecule for T cells

To gain insight on the T-cell functions that AMIGO2 may be involved in, we first performed bioinformatics analysis to identify *AMIGO2*-correlated genes in human blood T cells using microarray data stored in IST Online. The most significantly correlated genes ($p < 0.0075$) were then imported into DAVID for functional annotation clustering. Interestingly, among the *AMIGO2*-correlated genes, several clusters of genes are involved in organization of the cytoskeleton and extracellular matrix, and in regulation of cell migration (**III, Table S1**). Based on these data, and because AMIGOs are cell adhesion molecules themselves, we speculated that AMIGO2 may mediate T-cell adhesion.

To answer this question, we fluorescently labeled donor T cells enriched from the spleens of adult WT and AMG2KO mice with CFSE and eFluor 670, respectively, mixed them at a ratio of 1:1, and injected them intravenously into tail veins of WT recipient mice. After 15 h, we assessed the amounts of donor T cells (both Th and Tc) in the secondary lymphoid organs (including spleen, lymph nodes, and Peyer's patches) of recipient mice by staining with anti-mouse CD45-PE, CD8-PerCP/Cy5.5, and CD4-PE/Cy7 antibodies for flow cytometric analysis. As expected, AMG2KO donor Th and Tc cells had impaired capacity to accumulate into the secondary lymphoid organs of recipient mice (**III, Fig. 3**), suggesting that AMIGO2 functions as a T-cell adhesion molecule.

### 5.3.3. *Amigo2*-deficiency in T helper (Th) cells affects Th-cell activation, proliferation, and differentiation

We then systemically studied whether and how AMIGO2 might be involved in other major T-cell functions, including Th-cell activation, proliferation, and differentiation. For the Th-cell activation and proliferation assay, we enriched total Th cells from the spleens of adult mice and then applied appropriate stimulation, such as TCR co-stimulation (anti-CD3 with or without anti-CD28 antibody) or mitogen (ConA). After stimulation, *Amigo2*-deficient Th cells were hypo-

activated and hyper-proliferative as detected by flow cytometry (**III, Fig. 4**). The decrease in Th-cell activation but increase in proliferation due to the loss of *Amigo2* implies that AMG2KO Th cells may be less sensitive to TCR-induced cell apoptosis post-activation and are therefore more proliferative in response to anti-CD3 or ConA stimulation.

To explore whether *Amigo2*-deficiency may affect Th-cell functional polarization, we isolated naïve Th cells from the spleens of adult mice and then polarized them into Th1, Th2, and Th17 subtypes *in vitro* under respective polarization conditions (Th0 as a control condition for all other Th subtypes). After Th-cell differentiation, we quantitatively measured the protein levels of Th master transcription factors (T-bet for Th1 cells and GATA-3 for Th2 cells, respectively) in polarized Th cells by Western blotting. Interestingly, both T-bet and GATA-3 levels were elevated in AMG2KO Th1 and Th2 cells at 12 h and 24 h after *in vitro* polarization. With regard to Th17-cell differentiation, we performed intracellular cytokine staining with anti-IL-17A-Alexa Fluor 647 by flow cytometry and found that the percentage of IL-17A-producing Th cells was much lower in AMG2KO than WT controls (**III, Fig. 4**). We then measured Th-lineage cytokine levels in the culture supernatants at 72 h after *in vitro* polarization. In line with elevated T-bet and GATA-3 levels and decreased percentage of IL-17A-producing Th cells, AMG2KO Th cells secreted higher levels of IFN-γ and TNF-α under Th1, higher IL-10 under Th2, and lower IL-17A under Th17 conditions as compared to WT controls (**III, Table 1**).

### 5.3.4. *Amigo2* knockout (AMG2KO) mice exhibit ameliorated EAE severity accompanied with reduced spinal T-cell accumulation

The findings that *Amigo2*-deficiency led to aberrant T-cell functions, such as impaired T-cell accumulation into secondary lymphoid organs, hypoactivation, hyperproliferation, and skewed Th-cell differentiation, suggested that AMIGO2 might be involved in T-cell-mediated autoimmune diseases, such as EAE. We then subjected both WT and AMG2KO female mice to $MOG_{35-55}$-induced acute EAE and evaluated the clinical severity of paralysis daily from 7 dpi. Compared to WT controls, AMG2KO mice developed less severe EAE during 14-20 dpi.

To further understand EAE-related pathology in AMG2KO mice, we measured the number of infiltrating T cells (both Th and Tc cells) in the CNS (SC and brain) of EAE mice at 9 dpi by flow cytometry. In line with impaired accumulation of AMG2KO T cells into the lymphoid organs under steady-state conditions as previously observed, fewer Th and Tc cells infiltrated into the SCs of AMG2KO EAE mice compared to WT (**III, Fig. 5**).

### 5.3.5. Microglia/macrophages show anti-inflammatory response in the CNS of AMG2KO EAE mice

As EAE is a complex inflammatory condition in which both innate and adaptive immune cells are critically involved in the disease pathogenesis, we further studied the potential contribution from innate immune cells, including microglia and macrophages, and their inflammatory polarization states in the CNS of AMG2KO EAE mice. The total numbers of microglia or macrophages were similar between genotypes. However, when comparing their pro- and anti-inflammatory subtypes, we found that microglia in the brain, and macrophages in both the SC and the brain had lower M1/M2 ratios in AMG2KO EAE mice (**III, Fig. S3**), indicating anti-inflammatory properties of microglia and infiltrating macrophages in the CNS of AMG2KO mice under EAE conditions.

### 5.3.6. Anti-inflammatory response of splenocytes of AMG2KO EAE mice

To determine whether peripheral immune cells (particularly T cells), would have similar anti-inflammatory responses to EAE, we isolated EAE-sensitized splenocytes from WT and AMG2KO mice and assessed splenocytic recall responses by re-stimulating them with $MOG_{35-55}$ peptide *in vitro* for 72 h and measured cytokine levels thereafter using bead-based multiplex immunoassay. Although IFN-γ-production did not show any genotypic difference, IL-10 levels were significantly higher in the cell culture supernatants of AMG2KO splenocytes than WT (**III, Fig. 5**), indicating an enhanced anti-inflammatory response generated by AMG2KO splenocytes. Although both innate and adaptive immune cells might contribute to IL-10-production, we speculated that this effect was mainly conferred by T cells because we did not find that splenic macrophages showed any genotypic differences in M1/M2 macrophage ratios under both physiological and EAE conditions. The existence of anti-inflammatory microglia/macrophages in the CNS of EAE mice but not in the peripheral immune organs of either naïve or EAE mice was intriguing. We speculated that residing microglia and infiltrating macrophages in the CNS might be indirectly educated and reprogrammed by infiltrating anti-inflammatory Th cells that were primed in the periphery, thus cooperating with each other to confer neuroprotective effects and ameliorate EAE in AMG2KO mice.

The observations of elevated levels of IL-10 but not other canonical Th2 cytokines (IL-4, IL-5, and IL-13) under various experimental conditions (anti-CD3- plus anti-CD28-stimulated or Th2-polarized Th cells, or $MOG_{35-55}$ re-stimulated EAE-sensitized splenocytes) in AMG2KO mice

were also intriguing. It has been shown that IL-10 was more important than IL-4 in suppressing autoimmune Th1 responses in EAE by comparing IL-4- and IL-10-deficient or overexpressing mice (Bettelli et al., 1998). Besides, GATA-3 is capable of inducing directly *Il10* gene expression independently of *Il4* in Th cells (Saraiva and O'Garra, 2010; Shoemaker et al., 2006). Therefore, it is possible that upregulated GATA-3 due to *Amigo2*-deficiency induces IL-10 instead of IL-4, due to an unknown mechanism. Furthermore, several studies demonstrate that T-bet inhibits the expression of the Th17-specific transcription factor RAR-related orphan receptor gamma (RORγ)t, thereby resulting in suppressed Th17-cell differentiation, IL-17A production, and attenuated autoimmune responses (Fujiwara et al., 2007; Guo et al., 2009; Rutitzky et al., 2009). Elevated T-bet in AMG2KO Th cells may inhibit IL-17A production potentially via RORγt suppression. Therefore, we speculate that *Amigo2* inactivation reprograms differentiated Th cells to acquire an anti-inflammatory property with increased IL-10 (possibly via GATA-3) and decreased IL-17A production (possibly via T-bet-mediated RORγt inhibition).

Collectively, we demonstrated that *Amigo2*-deficiency impairs spinal T-cell accumulation and promotes anti-inflammatory innate immune responses in the CNS and peripheral immune organs, which may jointly contribute to ameliorated EAE severity in AMG2KO mice.

### 5.3.7. *Amigo2*-deficiency in Th cells promotes NF-kB and NFAT1 transcriptional activities via Akt activation

To understand the molecular mechanisms underlying AMIGO2-mediated regulation of T-cell signaling, we measured transcriptional activities of key transcription factors that are involved in TCR signaling, including NF-kB and NFAT1, by a dual luciferase reporter assay and a nuclear translocation assay. We observed that *Amigo2*-deficiency promoted transcriptional activities of NF-kB and NFAT1 (**III, Fig. 6**). Since Akt is a key kinase that regulates a variety of transcription factors, NF-kB and NFAT1 included, we further compared Akt activities between WT and AMG2KO Th cells under TCR co-stimulation and found that AMG2KO Th cells produced higher levels of phosphorylated Akt (**III, Fig. 6**). Taken together, these data suggest that *Amigo2*-deficiency promotes transcriptional activity of NF-kB and NFAT1, which may be achieved through Akt activation. Our hypothesized model of AMIGO2-mediated regulation of T-cell function is shown (**III, Fig. 6**).

In summary, using an *Amigo2*-deficient mouse line, we demonstrate that AMIGO2 is critically involved in the regulation of T-cell and microglial/macrophage functions, particularly in T-cell homing and inflammatory polarization of Th cells. More importantly, it has functional relevance

*in vivo* as loss of *Amigo2* resulted in ameliorated EAE severity accompanied by decreased spinal T-cell accumulation and enhanced anti-inflammatory responses in the brain, SC, and spleen. Mechanistically, we demonstrated that *Amigo2*-deficiency in Th cells enhances Akt activation and upregulates NF-kB and NFAT1 transcriptional activities, probably leading to elevated levels of T-bet and GATA-3 (and possibly decreased level of RORγt), consequently resulting in increased secretion of IFN-γ and IL-10 but decreased secretion of IL-17A. Our study thus suggest that AMIGO2 may be harnessed as a potential therapeutic target for treating MS.

However, it is important to note that in this study we used a conventional *Amigo2* knockout mouse model to explore its involvement in EAE, which is a complex condition in which a variety of immune- and neuropathological mechanisms are involved. Adoptive transfer EAE or a T-cell-specific *Amigo2* knockout mouse model is warranted in the future in order to exclude the potential contribution from neuronal-specific mechanisms that may confound our findings.

# 6. Conclusions and future perspectives

As the most versatile immune cells in the immune system, microglia/macrophages or T cells are implicated in various neurological and neuropsychiatric disorders. However, their precise inflammatory roles and molecular mechanisms underlying their regulation in the context of these disorders are largely unknown. In this thesis work, we first characterized the temporal-spatial roles of microglia/macrophages in chronic neuropathic pain and their possible contribution to analgesic efficacy or inefficacy of minocycline in rats. Secondly, we explored the association of brain microglial pro- versus anti-inflammatory activation with anxiety-like behaviors in mice. Lastly, using an *Amigo2*-deficient mouse model, we revealed novel roles of AMIGO2 in regulation of T-cell and microglial/macrophage functions, as well as its importance in acute EAE. The main conclusions and future perspectives are as follows:

1) Microglia/macrophages in the CNS are region-specific under steady-state conditions, in terms of their abundance, inflammatory polarization states, and expression of multiple microglia/macrophage-related immune genes. The CNS region-specificity of microglia/macrophages may determine their subsequent inflammatory responses to peripheral nerve injury and the analgesic effect of minocycline. Our data suggest that a future treatment strategy that targets not only spinal but also brain inflammation may be more powerful in alleviating chronic neuropathic pain. Importantly, our observation of a region-specific profile of microglia/macrophages unravels its critical role in the normal brain as well as in neurological or neuropsychiatric disorders.
2) Microglial M1/M2 ratios in the brain are positively correlated with anxiety-like behaviors in mice and rats. This suggests that brain microglial M1/M2 ratio may serve as an indicator of anxiety and a readout for diagnosis and screening of disease-modifying drugs for treating anxiety disorders or comorbid anxiety observed in various neurological patients. To unravel the molecular mechanisms of microglial polarization, the candidate genes found to be associated with microglial M1/M2 polarization should be validated with more specific genetic manipulation in animal models.
3) AMIGO2 modulates the functions of T cells and microglia/macrophages, particularly T-cell homing and pro- versus anti-inflammatory polarization of Th cells, and contributes to disease pathogenesis of EAE. Mechanistically, *Amigo2*-deficiency in Th cells promotes Akt activation and NF-kB and NFAT1 transcriptional activities, resulting in elevated levels of T-bet and GATA-3, possibly thereby leading to higher IFN-γ and IL-10 but lower IL-17A levels. Our findings suggest that AMIGO2 may be harnessed as a diagnostic and therapeutic target for MS. However, in this study, we used *Amigo2* global KO mice and several cofounding factors may mask the effect size of AMIGO2-mediated T cells in EAE. Therefore, future work is warranted to explore more specifically the role of AMIGO2 in T cells by using either adoptive transfer EAE or T-cell conditional KO mice.

In conclusion, using several rodent experimental models, we demonstrated critical contributions of pro- versus anti-inflammatory polarization of microglia/macrophages or AMIGO2-mediated Th cells in neurological and neuropsychiatric disorders. These findings provide novel insights into cellular and molecular mechanisms underlying neurological and neuropsychiatric disorders. Moreover, inflammatory polarizations of microglia/macrophages and Th cells, as well as the molecules that are critically involved in the modulation of their balance, may be harnessed as therapeutic targets for treating neurological or neuropsychiatric disorders.

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